



## Meeting the needs for released nanomaterials required for further testing - the sun approach

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*Total number of authors:*  
20

*Published in:*  
New tools and approaches for nanomaterial safety assessment - book of abstracts

*Publication date:*  
2017

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

### *Citation (APA):*

Nowack, B., Boldrin, A., Caballero, A., Hansen, S. F., Gottschalk, F., Heggelund, L. R., Hennig, M., Mackevica, A., Maes, H., Navratilova, J., Neubauer, N., Peters, R. J., Rose, J., Schäffer, A., Scifo, L., Leeuwen, S. V., Kammer, F. V. D., Wohlleben, W., Wyrwoll, A., & Hristozov, D. (2017). Meeting the needs for released nanomaterials required for further testing - the sun approach. In *New tools and approaches for nanomaterial safety assessment - book of abstracts* [#1515].

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7-9 Feb 2017  
Málaga



# New tools and approaches for **nanomaterial safety assessment**

## BOOK OF ABSTRACTS

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### **GUIDEnano: A tool for risk assessment of nanomaterials and nano-enabled products**

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Current uncertainties on the safety of nano-enabled products need to be urgently and carefully addressed. Otherwise, public fears could end up blocking the benefits of nanotechnology. Sound scientific information must be generated to identify potential risks of nano-enabled products on human and ecosystems health and, when considered unacceptable, efficiently mitigate such risks. This has to be done in a holistic manner, taking into consideration all stages of the life cycle of these products.

The main objective of GUIDEnano is to develop innovative methodologies to evaluate and manage human and environmental health risks of nano-enabled products, considering the whole product life cycle: Synthesis of NM, manufacturing of NM-enabled products, use, and end-of-life phase. These developments are incorporated into an interactive web-based Guidance Tool, which guide the NM-enabled product developers (mainly industry) and users into the design and application of the most appropriate risk assessment and mitigation strategy for a specific product. The correct implementation of this guidance ensures that the risks associated with a NM-enabled product, throughout its whole life cycle/ value chain, have been appropriately evaluated and mitigated to an acceptable level, according to the most recent knowledge at the time of implementation. The evaluation of a NM-enabled product using this Tool is also useful for risk communication to regulators, insurance companies, and society.

This presentation will show how GUIDEnano consortium worked together to achieve this main goal: overcoming specific goals, covering some gaps identified, implementing the current knowledge into a web-based Tool. Finally main messages on research needs will be given with the purpose to be taken over by the currently running/ recently starting/ future projects.

## **NanoSOLUTIONS keynote: Towards ENM Safety Classification**

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Nanotechnology has been recognised as a key enabling technology with significant economic and societal implications. Since safety is a prerequisite for the public success of nanotechnology it is critical to develop methods that give better prediction of the biological effects and risks of engineered nanomaterials (ENM) to humans and the environment. Simple, fast and cost efficient, and yet reliable methods are required to meet the challenge of the ever-decreasing time between the development of new ENM and their marketing. The overarching aim of the NANOSOLUTIONS project is to provide a means to develop a safety classification of ENM based on an understanding of their interactions with living organisms at the molecular, cellular, and organism levels. The objective is to determine the “biological identity” of ENM, and based on that, to develop a computational predictive tool for the assessment of ENM safety, i.e. ENM SAFETY CLASSIFIER. NANOSOLUTIONS project has generated set (n=31) of stable and well-characterized ENM and evaluated their exposure during several life-cycle stages. By using state-of-the-art models and approaches project has explored formation of ENM-biocorona and generated exceptionally large toxicological and ecotoxicological data set on ENM interactions with living systems. Various OMICS methods (microarrays, RNA-sequencing, proteomics) have been used to assess global expression levels of mRNA, miRNA and proteins from cells and organisms in response to all 31 NANOSOLUTION ENM. More importantly, project demonstrate for the first time how this large amount of high-quality data is used for generation of ENM SAFETY CLASSIFIER. In addition to proof-of-concept of ENM SAFETY CLASSIFIER project provides by far largest multi-layer OMICS data library derived from the robust cells and study organisms in response to large set of carefully characterized ENM. Computation predictive tool for assessment of ENM safety is an important step towards fast, reliable and affordable ENM safety classification.

## **SUN: EU project developing tools to facilitate assessment and management of nanotechnology risks**

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The rapid growth of the nanotechnology sector has raised not only high expectations, but also concerns about the health and environmental risks from engineered nanomaterials. The need to investigate these risks and find ways to prevent them became the reason to launch the large scale European FP7 SUN (Sustainable Nanotechnologies) project. With a budget of over 13 million EUR, its ambitious work program involved more than 100 scientists from 35 research and industrial organisations across 12 European states.

SUN developed reliable methods for characterization of nanoparticles released from products at different lifecycle stages into complex biological, environmental and food matrices, and for the assessment of their human and environmental exposure, hazard and risk. These tools and the newly developed safety by design procedures have become the highlights of the project and their integration into a decision support system and practical guidelines has provided industries and regulators with the means to streamline effective decision making about safer products and processes.

These newly developed methods and tools were validated in supply chains of real industrial products embedding nanoscale Tungsten Carbide (sintered, wear-resistant ceramics), Copper (antimicrobial/fungal wood preservatives), Silica (food), Titanium Dioxide (self-cleaning ceramic tiles and air purification systems), organic pigment (the red colour of the Ferrari cars), and multi-walled carbon nanotubes (anti-fouling coatings, lightweight plastics). This approach not only generated an enormous amount of new scientific data and knowledge but also practical guidelines for risk management and safer product and process design.

# eNanoMapper: Advancing a Sustainable Knowledge Infrastructure for the Safety Assessment of Nanotechnology

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Establishing standards and setting up harmonised infrastructures with applicability in nanosafety, still represents a major challenge for the scientific community. The complexity of the toxicology, chemistry and biology and the additional physicochemical properties of nanomaterials, leads to increased uncertainty in the validity of experimental data. To support these international efforts, eNanoMapper delivered an ontology, a data infrastructure and modelling tools with applicability in risk assessment of nanomaterials. The infrastructure developed for nanomaterials toxicological data is based on open standards, open source, common languages, and an interoperable design, enabling a more effective and integrated approach in risk assessment (see Figure 1). The main achievements of eNanoMapper towards improved standards in risk assessment of nanomaterials and with an impact on the nanosafety community are represented by an agreed language formalized in a nano ontology [1], an open platform for integrating different nanomaterials data sources providing access to open and confidential data [2] and the computational infrastructure, analysis and modelling tools for predicting toxicity of nanomaterials [3]. The eNanoMapper approach supports the integration of non-testing methods into risk assessment [4], and facilitates a harmonised use of existing data and knowledge, enabling a significant reduction of animals used for nanomaterials toxicity testing [5]. The project also provides a rich library [6] of information and documentation (tutorials, webinars, reports and publications) to support and guide the users.

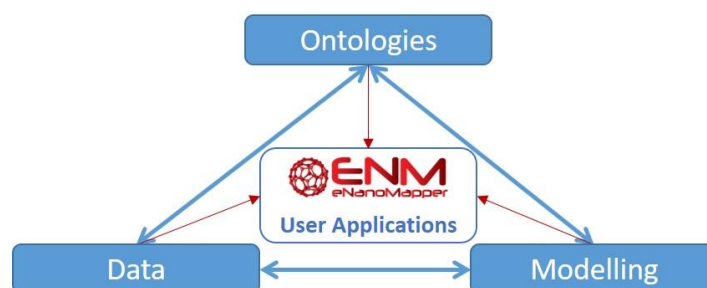


Figure 1: eNanoMapper integrative approach towards improved standards in risk assessment of nanomaterials

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# NanoMILE: Towards mechanistic understanding of nanomaterials interactions: a hazard assessment framework

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Despite being relatively new, nanoscience and nanotechnology have advanced rapidly in terms of generating scientific discoveries along with commercial applications. However, the field of nanosafety, which is the science of assessing hazards and risks from novel nanomaterials, has not kept pace with these developments. Potentially the greatest concern in the science of nanosafety is the lack of a paradigm for MNM mode of action, which necessitates that each MNM is considered individually for its hazard and risk. This is coupled with constraints within REACH regulation which currently allows read-across only within an existing family of chemicals, so in the case of nanomaterials for example, from one titania nanomaterial to another, rather than within families of nanomaterials, e.g. metals or metal oxides. NanoMILE's overarching aim was to develop detailed mechanistic understanding of the interactions of manufactured nanomaterials (MNM) with living systems as the basis for predictive tools for nanomaterials hazard assessment. Test systems in NanoMILE ranged from biofluids, simple unicellular through to multi-cellular organisms, whole animals and humans. Identification of conserved pathways across species and development of *in vitro* alternative test methods and high throughput approaches is a core aim, in line with the European Commission's drive to reduce animal testing (the 3Rs) and to ensure safe and responsible implementation of nanotechnologies. In order to support the design of safer MNMs, the work in NanoMILE involved a central iterative link between MNM properties and biological/environmental effects, in order to understand, for example, if certain features of the particles were clearly identified as inducing toxicological effects, whether these features could be "*designed out*" while keeping all other parameters and the core functionality intact as far as possible. The opposite strategy was also investigated: whether it is possible to take inherently inert particles and "*design-in*" toxicity, for example tailoring bandgap to introduce redox activity or oxidative stress, as a means to conclusively verify the mode of action as being related to that physicochemical property or descriptor. The ultimate outcome from NanoMILE is a framework for hazard categorisation and a set of tools including QSARs and QPARs (as it can be physicochemical "properties" or specific "structures" linked to a specific mode of hazardous activity). The models are based on the largest documented set of nanomaterials, comprising the NanoMILE libraries of particles which consists of around 200 nanomaterials, with complete characterisation datasets for 96 of these) spanning the broad materials families of metals (silver, gold), metal oxides (titania including a range of different coatings, ceria including a series of zirconia doped cerias, zinc, copper) and carbon nanomaterials, and incorporate high content screening data of a series of end-points such as from cells and zebrafish embryos. These are being enriched with *in vivo* toxicity data (mice/rats and a range of environmentally relevant species including algae, daphnia, zebra fish, *C. elegans*, isopods etc.) as well as with metabolomics and transcriptomics data, and corona composition for a sub-set of the nanomaterials. This dataset (the NanoMILE KnowledgeBase), and the mechanistic understanding obtained from interrogating and integrating it, is enabling important steps towards safer by design nanomaterials, and predictive classification and grouping of nanomaterials potential hazard on the basis of their key physico-chemical descriptors, as well as feeding into the development of Adverse Outcome pathways and regulatory decision making regarding nanomaterials.





**#1511**

**Detection, quantification and identification of engineered nanoparticles in complex media at ppb-concentrations**

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Permanent growth in the production of engineered nanoparticles increases a risk of human contacts with these potentially hazardous materials. It makes important a development of sensitive analytical methods for quantification and identification of nanoparticles in liquid and gaseous media. For this purpose, a method based on the detection and analysis of minute signals of surface plasmon resonance (SPR) images due to adsorption of single nanoparticles was developed. This new technology allows one a real-time detection of interaction of single nanoparticles with sensor surface. Adsorption of each nanoparticle leads to characteristic diffraction image whose shape and intensity depends on the size and chemical composition of nanoparticle. A number of the nanoparticle–surface binding events per time and surface area characterizes volume concentration of nanoparticles. A large monitored surface area of sensor surface allows one to detect many hundreds events in each frame, this leads to a very high dynamic range of counting and to a correspondingly high dynamic range in the concentration scale. Depending on the type of nanoparticles and experimental conditions, the detection limit for aqueous samples can be below 1000 nanoparticles per microliter. Statistical analysis of images of nanoparticles provides information on heterogeneity of nanoparticles and can be used as fingerprints for identification of different types of nanomaterials. Chemical functionalization of the sensor surface as well as changes of pH or ionic strength are additional factors influencing the behavior of nanoparticles and allowing one their identification. Independent information on chemical composition and size of nanoparticles can be obtained from SPR visualization of their electrochemical dissolving or modification during potential sweep. The method was also applied for ultrasensitive detection and analysis of nanoparticles in very complex media, such as tap water, sunscreen, juice or wine.

Beside analytical applications, the new approach provides valuable scientific information on adsorptive properties of nanoparticles which can be used to predict their toxicity.

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# GOODNESS OF DUSTINESS INDEX FOR PREDICTING HUMAN EXPOSURE TO AIRBORNE NANOMATERIALS

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Human exposure modelling usually require quantitative process-specific release data and emission characteristics in the potential exposure scenario [1].

In this particular study, occupational exposure levels of a paint industry during powder pouring processes of paint pigments/fillers from small (25 kg) and big (500 kg) bags [2] and in a laboratory during nanodiamonds sieving and handling [3] were assessed in the near field (NF) and far field (FF) and compared with the measured levels in laboratory experiments (Table 1). Particle concentration measurements and gravimetric PM sampling were performed. In addition, particle morphology were characterised by scanning and transmission electron microscopy. Dustiness indices for the specific powders were characterized by using a down-scaled EN 15051 dustiness rotating drum [4] and applied in two-box model NF/FF [5] and ART model [6] to predict exposure mass concentrations [2]. The source emission rates were characterised by dustiness indices ( $D_{lm}$  in  $\mu\text{g kg}^{-1}$ ) and indoor aerosol modelling [7] by taking into account a handling energy factor and a localized control factor.

An overestimation ranging from 21-78 % was obtained for all the processes excepting the pouring process of a small bag which was underestimated. This underestimation suggests that the used dispersion model did not seem to work properly when pouring small amounts of  $\text{TiO}_2$ . To improve the predictability in modelling, a better understanding is needed in regards to using dustiness data in exposure modelling.

Table 1. Process-specific release data and emission characteristics during sieving and handling processes.

	Handling nanodiamonds			Pouring $\text{TiO}_2$ RD3	
	Sieving in a fume hood	Sieving in a room	Cleaning	500 kg bag	25 kg bag
Emission rates [ $\mu\text{g min}^{-1}$ ]	1.9	15.2	4.0	870	1700
Mass measured [ $\mu\text{g m}^{-3}$ ]	0.24	4.96	1.54	0.08	0.17
Mass NF modelled [ $\mu\text{g m}^{-3}$ ]	0.78	6.27	2.70	0.37	0.10
Mass FF modelled [ $\mu\text{g m}^{-3}$ ]	0.16	1.19	1.17	-	-

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## LAB-SCALE SIMULATIONS TO QUANTIFY NANOMATERIALS RELEASE FROM NANO-ENABLED PRODUCTS

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During the common use of products containing nanomaterials (NMs), namely nano-enabled products, these can spread and be exposed to uncontrollable conditions, increasing their potential impact on humans and the environment. Over the last decade, NMs use and diversity of applications have grown extensively and continue to grow rapidly. Therefore, NMs release from commercial products must be identified and studied to adequately prevent and treat any eventual release to the environment, especially if those NMs suppose a risk.

The present work aims at evaluating NMs environmental release from several real nano-enabled products proposed by the industrial partners in GUIDEnano project, using experimental tests at lab scale simulating the intended use of the products. Several examples of products containing adsorbed NMs (e.g. photocatalytic coatings on roads and ceramics) and products with embedded NMs (e.g. polymeric nanocomposites and antifouling paints) will be presented. Such case studies cover reactive/inert NMs and the different potential routes to the environment, such as WWTP, soil or water compartments. According to their specific use, these materials have been exposed to different processes, namely i) weathering in climatic chamber, ii) washing processes, and iii) liquid contact scenarios. Comprehensive physico-chemical characterization of both starting and materials after use simulation has been performed, and especial emphasis has been put on analytical techniques used to monitor NMs release and matrix transformations (e.g. FTIR, XPS or electron microscopy).

Results obtained can be used to feed existing environmental exposure models by refining current environmental release factors, also giving insights into relevant exposure forms and whether release NMs keep their original characteristics. Moreover, results also shed light into the mechanisms which promote NMs release. Finally, release assessment can contribute to the industrial development of safe products by tailoring ENM properties and modifying matrix – NMs interactions. Several examples will be provided on how to decrease photocatalytic activity of NMs or increase the attachment efficiency of NMs on surfaces.

The authors gratefully acknowledge the support of this research by the European Commission within the Seventh Framework Programme (FP7/2007-2013), Grant Agreement 604387 (GUIDEnano).

## **HOT SPOT RELEASE MAPPING OF NANOMATERIALS – A VISUAL EXPOSURE ASSESSMENT METHOD FOR PRELIMINARY ASSESSMENT**

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The development of nanomaterials especially of the next functionalized generation is still in an early phase of development. In these early phases, we are confronted with the Collingridge dilemma between far reaching design options on the one hand and a very limited availability of reliable knowledge about expectable impacts (Collingridge 1980). When products, processes, applications and their contexts are still unknown or uncertain the assessment must focus on what is already knowable, this is the character of the technology, of the materials and their functionalities and the intended applications based on them. The characterization of the materials and their functionalities offers information about hazard and exposure potentials (e.g. mobility in the environment, persistence etc.).

Based on this information the contribution will present a preliminary visual exposure assessment method. The tool integrates expert knowledge and literature data about release points and release quantities and extends the modeling approach to the entire life cycle of the nanoapplication.

Interviews of expert answers to questionnaires and literature survey build the basis for a detailed visualization of the life cycle stages of nanoapplications as well as for the weighting of release potentials. Based on this hot spots maps are created in which the environmental releases potentials are marked with an arrow and the weight of the flows corresponds with the thickness of the lines.

Thus the relevant nanospecific release potential can be easily identified along the life cycle stages of the nanoproduct and this knowledge may be used in the process of a precautionary product development and product use optimization. Some examples of hot spots release maps developed in the SUN project will be presented.

## MEETING THE NEEDS FOR RELEASED NANOMATERIALS REQUIRED FOR FURTHER TESTING – THE SUN APPROACH

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The analysis of the potential risks of engineered nanomaterials (ENM) has so far been almost exclusively focused on the pristine, as-produced particles. However, when considering a life-cycle perspective, it is clear that ENM released from genuine products during manufacturing, use, and disposal is far more relevant. Research on release of materials from nano-products is growing and the next necessary step is to investigate the behavior and effects of these released materials in the environment and on humans. Therefore, sufficient amounts of released materials need to be available for further testing. In addition, ENM-free reference materials are needed since many processes not only release ENM but also nano-sized fragments from the ENM-containing matrix that may interfere with further tests. The SUN consortium (Project on "Sustainable Nanotechnologies", EU 7th Framework funding) uses methods to characterize and quantify nanomaterials released from composite samples that are exposed to environmental stressors. Here we describe an approach to provide materials in hundreds of gram quantities mimicking actual released materials from coatings and polymer nanocomposites by producing what is called "Fragmented Products" (FP). These FP can further be exposed to environmental conditions (e.g. humidity, light) to produce "Weathered Fragmented Products" (WFP) or can be subjected to a further size fractionation to isolate "Sieved Fragmented Products" (SFP) that are representative for inhalation studies. In this perspective we describe the approach, and the used methods to obtain released materials in amounts large enough to be suitable for further fate and (eco)toxicity testing. We present a case study (nanoparticulate organic pigment in polypropylene) to show exemplarily the procedures used to produce the FP. We present some characterization data of the FP and discuss critically the further potential and the usefulness of the approach we developed.





## EXPERIMENTAL LIFE-CYCLE SIMULATIONS OF NANO-ENABLED PRODUCTS AND CHARACTERIZATION OF RELEASED MATERIALS

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This work has been done within the framework of the NANOSOLUTIONS FP7 European research project, to study the release of ENMs along their life cycle when used in existing applications. Firstly, the life cycle stages in which are most likely to result transformation of ENMs and/or to result in the release of ENMs have been identified, for seven products, prioritizing normal use conditions of products of commercial relevance. Relevant processes in terms of engineering nanomaterials (ENM) transformation and/or release during the use and end-of-life stages of nano-enabled products have been experimentally simulated. Textile washing (CuO, TiO<sub>2</sub> and Ag), oil aging inside a car engine (nanodiamonds), ink printing (CdTe Quantum Dots (QD)), transformations in human physiological fluids of gold nanocarriers and mechanical abrasion on knitted polymeric materials (MWCNT) have been performed. Nano-enabled products in the project cover different ENM distribution into products: 1) ENM adsorbed on surfaces, 2) ENM embedded within polymeric matrices, and 3) ENM dispersed in different solvents.

In order to monitor the transformations and particle properties, both pristine and released particles have been analyzed and compared. Size and aggregation state were characterized by TEM and DLS, and surface charge by means of zeta potential measurements (for stable dispersions). Moreover, the wt% of organic coatings or surface impurities attachment was determined by thermogravimetric analysis (TGA), Raman spectroscopy, IR and/ or elemental analysis. Finally, ionic release experiments have been conducted to determine the content of ions in the receptor solution by ICP-MS.

Release potential and exposure relevant forms have been determined for each one of the simulations evaluated, as well as the different receptor environmental compartments. Substantial ENM release has been observed in two cases studies: The waters collected from the washing experiments (Textiles) and also into the air due to the CdTe particles emitted from a conventional printer (Inks).

The authors gratefully acknowledge the financial support of this research by the European Commission within the Seventh Framework Programme (FP7/2007-2013), Grant Agreements 309329 (NANOSOLUTIONS).

## SWIMMING PERFORMANCE AND BEHAVIOURAL CHANGES IN ZEBRAFISH (*DANIO RERIO*) AFTER EXPOSURE TO NANOMATERIALS

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*Danio rerio* (zebrafish) is being established as a model organism for applications in several fields of research forming a link between cell culture models and mammalian models. The advantage of a rapid reproduction cycle permits zebrafish bioassays to be studied all the way from embryo level up until adult each with different end points (Goldsmith & Solari, 2003; Westerfield, 2000). The tests are also relatively rapid, inexpensive and straightforward to perform (Shaw and Handy, 2011; De Jong *et al.*, 2008). The close homology between the human genome and the zebrafish genome make it a model candidate for behavioural and sub cellular toxicity testing, the genetic parallels include physiological and anatomical similarities between the endothelial cells, blood brain barrier, social interactions and immunogenic responses (Fako and Furgeson, 2009). In an effort to gain a better understanding of the effect nanomaterial exposure has on *D. rerio* a novel system being used for neurobiology was employed to study changes in animal behavior as an indicator of stress. The Noldus system tracks the activity of the fish within an arena using the digital midpoint marker by highlighting the fish from the background. Adult zebrafish which were housed in a Tecniplast Zebrafish housing system at 26°C were transported to a behavioral room kept at 26°C and individually placed into 1.1L Tecniplast tanks in front of a frontal camera setup. The *D. rerio* acute lethality test (OECD-TG203) was performed on adult fish. Three replicates consisting of four adults each were carried out in 1.1L Tecniplast tanks and oxygen saturation was maintained above 60% for the duration of the test. Video parameters were set to record at 25 frame rates per second using adult fish digitally marked at their center point. Fish were tracked simultaneously in different tanks falling into different arenas where an upper and lower section was marked on each arena to determine where fish spent most of their time, arenas were scaled according to the dimensions of the tank (upper and lower). Fish were left undisturbed for the duration of the recordings. Video recording were interpreted by physically viewing them and interpolating any missing data points by placing the marker on the animal in the coordinates it appeared in the arena as well as reassigning any incorrect data points where the software had lost sight of the organism. Exposure concentrations were compared to a control group and several parameters were assessed; these included mean velocity, distance moved, zone transitions, cumulative mobility and meander. Data was imported to GraphPad and statistics were determined using One Way ANOVA's to compare fish over time and temperature change with Tukey's post hoc tests, significance was seen where  $p < 0.05$ . The nanomaterial exposure groups had decreased average velocity and distance moved which in turn showed less zone transitions when compared to control groups. The changes in behaviour of fish was concentration dependent where lower concentrations were more affected possibly due to nanoparticle agglomeration within the exposure medium which is a limiting factor for body uptake by the organism.

## THE SUN TIERED MODELING-BASED INHALATION, DERMAL, ORAL AND INADVERTENT ORAL EXPOSURE ASSESSMENT FRAMEWORK FOR NANOMATERIALS

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As part of the EU FP7 SUN project, we set out to establish a tiered modelling-based framework for the nanospecific inhalation, dermal, oral and inadvertent oral exposure assessment to manufactured nanomaterials and nano-enabled products. The first task was to define which additional information- and model properties the assessment of nanomaterials would require considering the different exposure routes. Second, the output must match the data generated during hazard assessment to enable integrated risk assessment.

Assessment of exposure characteristics revealed that knowledge on size-distributions was highly important for assessment of airway exposure. Also in dermal risk assessment, the particle sizes may play a role for very small nanoscale NOAA.

It was found that despite new metrics for hazard assessment is often discussed; the hazard assessment results remained to be based on mass-concentrations. However, there remains a wish for additional information on particle size-distributions and surface area of which measurement of surface area exposure can be associated with great uncertainty.

The SUN exposure assessment framework was consequently established considering a suite of existing available and new nano-specific models and tools for each of the exposure routes considered.

- Oral exposure assessment is modelled using the simple TRA and ConsExpo models for both consumers and workers.
- Inadvertent exposure is modelled using a modified version of the IEAT model, which is modified for assessment of nanomaterials.

- Dermal exposure assessment is proposed to be given using ConsExpo and for sprays and powders coupling between the deposition in function in dART the inhalation exposure assessment models below, which allows a size-distribution specific assessment.
- Inhalation exposure assessment is provided using the NanoSafer single- and two-box models and the ConsExpo Nano model at lower tier and a new multi-box model for higher tier exposure assessments. Functions to take aerosol dynamics into account have been added to the two-box models to enable a more predictive estimate of the exposure concentrations.

An overall requirement in this exposure assessment framework is that all assessments are based on availability of mass-based nanomaterial concentrations, release- and transfer rates or –efficiencies to assess exposure to releases from powders, sprays, mechanical reduction, dispersions/formulations, material contact, and food. The presentation will include examples of exposure assessments along the product value chain with primary focus on the SUN case studies and comparison with measurement results.

The authors acknowledge the EU Commission FP7 program for funding to the SUN project (Grant agreement No: 604305), which made this work possible.

## EXPOSURE ASSESSMENT OF GUIDEnano CASE STUDIES

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Within the GUIDEnano project data on occupational inhalation exposure and release was generated for eight case studies in the following sectors : construction (TiO<sub>2</sub>); textile (nano-Ag); food industry (nanocellulose); automotive (CNT); paints (ZnO); consumer goods (Al<sub>2</sub>O<sub>3</sub>-SiO<sub>2</sub> & TiO<sub>2</sub>) and environmental remediation (FeO).

Hot spots for emissions of engineered nanomaterials (ENMs) were identified and a cost-effective strategy for exposure assessment was followed. The strategy followed a three-tier approach where the level of detail of the assessment increases and uncertainty decreases with the tier. We started on tier one and evaluated the available information to decide whether to proceed with tier two (when we considered emission was likely but exposure was possibly low) or tier three (when we considered exposure was likely and potentially moderate/high or the ENMs were considered of high risk for human health) or to stop there (when we considered exposure was unlikely). Measurement data was evaluated for the likelihood of exposure following a decision logic developed by Bekker et al (2014).

A total of 24 occupational exposure scenarios were evaluated: Tier three was applied to nanocellulose (1 scenario) and CNT (2 scenarios); tier two was applied to TiO<sub>2</sub> used in construction (3 scenarios) and TiO<sub>2</sub> in paints (2 scenarios); nano-Ag (6 scenarios); ZnO (8 scenarios) and Al<sub>2</sub>O<sub>3</sub>-SiO<sub>2</sub> ( 2 scenarios). For FeO the evaluation stop in tier one (1 scenario). Exposure was likely for the scenarios involving CNT, TiO<sub>2</sub>, ZnO and Al<sub>2</sub>O<sub>3</sub>-SiO<sub>2</sub> and unlikely for the scenarios of nano-Ag, FeO and nanocellulose. The decision to move to tier 2 or tier 3 requires some further refinement.

The authors would like to acknowledge the EU Commission FP7 program for funding this project (Grant agreement No: 604387).

Bekker C, Brouwer DH, van Duuren-Stuurman B, Tuinman II, Tromp P, Fransman W. (2014) Airborne manufactured nano-objects released from commercially available spray products: temporal and spatial influences. *JESEE*; 24: 74-81.

**#1532**

**Life-cycle inspired protocols for aging engineered nanomaterials: Applicability of comparing pristine and transformed particles to understand nanoparticle behavior and (eco)toxicity**

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The use of pristine, as-manufactured nanomaterials (MNM) to examine environmental behavior or toxicity is convenient, but it is the aged MNM that are much more likely to be released into the environment and it is these materials which organism will ultimately be exposed to. When determining which aging procedures are most relevant, a life cycle perspective can be useful to gain insight into how MNM will be used, released from products and move through technical and environmental systems. While there are a few pristine nanomaterial standards or test materials, procedures to create appropriate aged MNM standards are more rare. In this study, we propose standardized aging procedures representing various stages of the product life cycle for a selection of common MNM (CeO<sub>2</sub>, TiO<sub>2</sub>, ZnO and Ag). Significant chemical and physical transformations are presented in the results; ranging from changes in speciation, to surface coatings, to agglomeration and changes in surface charge. Furthermore, we present the implications of using pristine MNM versus these aged MNM in test systems. In conclusion, various aging paradigms that may be followed are presented, each of which comes with pros and cons in terms of how close the aging parameters are to the real world. The relative difficulties with following each path of material choice are also discussed. Therefore, this work deals with both the practical laboratory work to produce relevantly aged MNM and the theoretical backing as to why such work is necessary. Because of the ubiquitous necessity for all researchers using MNM in environmental or ecotoxicity testing to use materials that are most likely to be in the environment, these conclusions of how product use and release related MNM aging and transformations may affect toxicity is an important result from the nanoMILE project.

## A KINETIC ENVIRONMENTAL FATE MODEL FOR THE RISK ASSESSMENT OF ENGINEERED NANOMATERIALS

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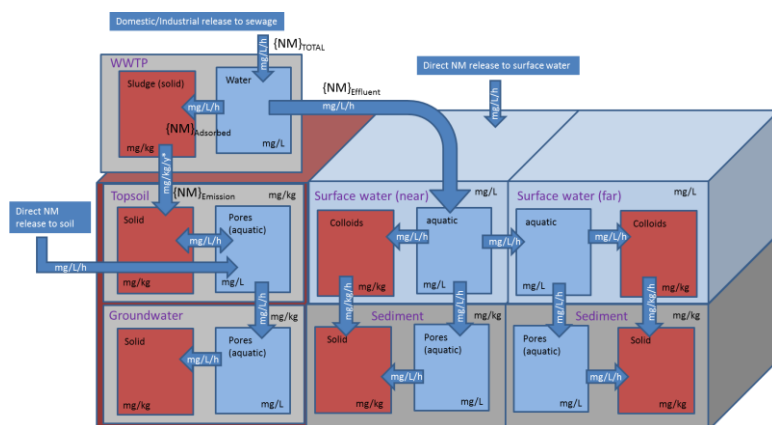
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An integrated exposure model is proposed for the prediction of the environmental fate of nanomaterials (NMs) in various natural and man-made compartments. A semi-mechanistic approach is used, wherein the kinetic nature of the NM fate processes takes focus. Environmental matrix interactions depend on NM properties and relevant parameters, such as organic matter and ionic conditions, and are calculated on a temporal scale in each compartment. The obtained NM bioavailability is afterwards linked with existing hazard information. The goal of this approach is to apply the model for most currently-produced and possible future NMs, also aiming to find a compromise between mechanistical accuracy and operational simplicity.



**Figure 1:** Schematic overview of the model world approach. Units in the compartments are time-dependent mass concentrations, whereas arrows represent fluxes between zones and compartments.

The current model gives fate descriptors for soil, surface water, and wastewater treatment plant (WWTP) experimentally validated, including also rates for key processes such as dissolution, sulfidation, heteroaggregation and sedimentation. A time-dependent approach is given to prioritize these key processes.

The WWTP is the main entry point for NMs emitted with domestic and industrial liquid waste. Partitioning of NMs in the WWTP, based on their affinity to sludge, determines the amounts that are transported to the surface water (in the effluent) and the soil (via sludge).

The physicochemistry of the surface waters, and the properties of the NM are among the factors that affect its bioavailable concentration in the water column and, after possible sedimentation, in the sediment within a certain time.

In the soil environment, focus is given on the top layer, where the NMs arrive also via the annual deposition of sludge. It is also in this layer where the most bioactivity and potential toxicity will occur. The time-dependent concentration in the pore water (liquid phase) and on the soil material (solid phase) is determined using a dual deposition model, allowing both NM release and irreversible attachment.

The time-dependency of chemical species concentrations is essential since it links exposure scenarios with hazards that change over time, which is critical in assessing NM bioavailability and eventual risks. This kinetic fate model is a key component in the web-based tool for the assessment and management of risks associated with NM-enabled consumer products, under the GUIDEnano Project (EU FP7).

The authors gratefully acknowledge the support of this research by the European Commission within the Seventh Framework Programme (FP7/2007-2013), Grant Agreement 604387 (GUIDEnano).



## ASSESSMENT OF DIETARY UPTAKE OF COPPER OXIDE NANOMATERIALS IN RAINBOW TROUT USING AN IN VITRO GUT SAC TECHNIQUE

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Few studies have considered dietary uptake of nanomaterials (NMs) in fishes despite physicochemical data of NM behaviours in aquatic ecosystems suggesting the diet may be an important exposure pathway; NMs may be predicted to settle out of the water column and associate with benthic organisms which may be food to fishes. OECD Test Guideline 305 includes procedures for assessing the exposure of fishes to substances in the diet but the long duration of these tests (4-8 weeks) means that they are uneconomical for assessing the dietary accumulation potential of the large numbers of NMs awaiting ecotoxicity testing. Here, we propose a tiered approach to dietary exposure assessment by using high-throughput *in chemico* and *in vitro* assays as initial pre-screening methods to identify NMs of concern before an *in vivo* dietary feed trial is considered.

In this paper we present example data for pristine uncoated copper oxide nanomaterials (CuO NMs) in rainbow trout (*Oncorhynchus mykiss*). We performed dissolution analysis of the CuO NMs in a simplified physiological saline adjusted to acid pH to simulate digestion in stomach of trout and to identify a rapid bioaccessible fraction of the CuO NMs. After 4 h, dissolution of CuO NMs was  $0.36 \pm 0.01$  % at pH 7.8, but this increased to  $27.6 \pm 8.3$  % at pH 5 and  $77.9 \pm 8.3$  % at pH 2. These data indicate CuO NMs undergo rapid dissolution at low pH with implications for bioavailability of Cu/ CuO NMs in the intestine. An *in vitro* screen of dietary bioavailability of CuO NMs was performed in rainbow trout using an established gut sac technique. Adult rainbow trout were euthanised and the whole gastrointestinal tract removed and separated into stomach, anterior, mid and hind intestine regions. Tissues were rinsed through with saline and then filled with  $3.175 \text{ mg L}^{-1}$  of Cu as  $\text{CuSO}_4$  or as pristine CuO NMs prepared in saline at pH 7.8. Additional control gut sacs were filled with saline, only. After tying both ends with suture thread to form the sac, tissues were incubated in saline continually gassed with 99.7%  $\text{O}_2$ : 0.3%  $\text{CO}_2$ . After 4 h, tissues were rinsed to remove loosely bound Cu and then deconstructed into enterocyte and muscularis layers. Copper concentrations were then measured with ICP-MS. Copper was elevated in enterocytes of mid and hind intestines of trout exposed to  $\text{CuSO}_4$  compared to controls. Elevations in Cu were also recorded in the underlying muscularis layer indicating Cu as  $\text{CuSO}_4$  is bioavailable to trout and exposure at  $3.175 \text{ mg L}^{-1}$  will likely lead to accumulation in internal tissues. In contrast, increases in Cu concentrations were not measured in trout tissues exposed to CuO NMs, indicating most was surface bound and CuO NMs have low bioavailability in trout. However, given that the *in chemico* data obtained indicate CuO NMs undergo dissolution at acid pH, a full *in vivo* exposure in trout would be required to assess dietary uptake potential of CuO NMs. This research is a contribution to EU FP7 SUN Project.

## INTEGRATION OF ECOTOXICOLOGICAL EFFECTS OF ENMS INTO LIFE CYCLE IMPACT ASSESSMENT

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Life cycle impact assessment (LCIA) methods describe environmental impacts in terms of characterization factors. Even if it has been claimed that LCA is an essential tool to analyze, evaluate, understand and manage the environmental and health effects of nanotechnology, few studies integrate the environmental impacts of engineered nanomaterials (ENMs). Characterization factors (CFs) for freshwater ecotoxicity need to be developed to make LCA into a practical tool, particularly for ENMs.

Within the NANOSOLUTIONS Project, a LCA assessment of a water based printing ink containing polyethyleneglycol-coated (PEG-CdTe) quantum dots (QDs) manufactured by Plasmachem is being carried out. The present assessment integrates specific c CF for CdTe QDs that are actually being developed. This work goes beyond the state-of-the-art developing not only CFs for CdTe QDs pristine but also for CdTe QDs released.

To this aim, following the USEtox® model<sup>1,2</sup>, which is the the UNEP-SETAC consensus methodology for Freshwater Ecotoxicity and Human Toxicity characterization factors calculation to be used in Life Cycle Impact Assessment, the ecotoxicological effects of CdTe QDs are being investigated. Both analyzing the

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<sup>1</sup> Rosenbaum, R.K., Bachmann, T.M., Gold, L.S., Huijbregts, M.A.J., Jolliet, O., Juraske, R., Koehler, A., Larsen, H.F., MacLeod, M., Margni, M., McKone, T.E., Payet, J., Schuhmacher, M., Van De Meent, D., Hauschild, M.Z., 2008. USEtox - The UNEP-SETAC toxicity model: Recommended characterisation factors for human toxicity and freshwater ecotoxicity in life cycle impact assessment. International Journal of Life Cycle Assessment 13, 532-546

<sup>2</sup> Official page of the USEtox model: <http://www.usetox.org/>

outcomes of the experimental work carried out within the project and completing these results with data from the existing literature.

Having evaluated the ecotoxicological effects of CdTe QDs for their integration into LCIA from the sources described above, a series of limitations have been observed. Such limitations are evaluated so that the outcomes of future studies on QDs freshwater ecotoxicity can be used to such purpose. Possible adaptations of the USEtox® model for freshwater effects CF development for ENMs are also suggested.

**This research received support from:**

**The NANOSOLUTIONS Project which has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement #309329.**

## LIFE CYCLE THINKING OF NANOTECHNOLOGY BASED APPLICATIONS

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As part of the EU FP7 SUN project, we implement the life cycle perspective in the SUN case studies. In order to assess potential environmental hot spot releases and environmental lifecycle impacts, the life cycle assessment (LCA) methodology has been applied and the results have been compared to conventional products with similar uses and functionality.

The project SUN has selected and has investigated the specific nanomaterials and associated products during the life cycle of the products (preproduction, production, use phase, end-of-life and recycling phases (re-use, recycling and/or final treatment and disposal) in different case studies (CS):

- Nano-WC-Cobalt (Tungsten Carbide-cobalt) sintered ceramics
- Nanocopper wood preservatives
- Carbon Nano Tube (CNT) in plastics
- Silicon Dioxide (SiO<sub>2</sub>) as food additive
- Nano-Titanium Dioxide (TiO<sub>2</sub>) air filter system
- Organic pigment in plastics
- Nanosilver (Ag) in textiles

The variety of investigated nanomaterials and applications are very different and so also the results of the LCA case studies. In some case studies, the environmental impacts from nanomaterials are low, in other case studies, however, significant. No clear statements to the environmental benefits are possible when compared to conventional products. Environmental impacts of the production of nanomaterials depend on the type of manufacturing process (energy demand, demand of operating supplies, yield, purification rate). In the case studies we can see a great range of factors of environmental impacts of the production of nanomaterials in comparison with micro-sized or conventional materials.

The presentation will present examples of the results of hot spot releases mapping and of life cycle assessments on the SUN case studies and will discuss the following questions: What is the environmental impact of the production of nanomaterials? What is the influence of these nanomaterials on the environmental impact of new (prospective) applications? Which kind of nanoapplications we need in future to realize high environmental (sustainable) benefits?

The authors acknowledge the EU Commission FP7 program for funding to the SUN project (Grant agreement No: 604305), which made this work possible.

#1564

## DISSOLUTION TEST FOR RISK ASSESSMENT OF NANOPARTICLES: A PILOT STUDY

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Nanotechnology is one of the major scientific revolutions that the food industry has experienced over the last years. In particular, the agro-food market has exploited the well-known antimicrobial activity of silver nanoparticles (AgNPs), employing them as food additives and food contact materials to extend the shelf life of consumer products.

Since the human ingestion by voluntary and involuntary actions is possible, the need to get information about the possible adverse effects raised by nanoscale silver on health is therefore urgent. And the knowledge of fate and identity of the molecular species arising from AgNPs biotransformation process in human biological fluids is fundamental.

This argument is currently under extensive debate by regulatory agencies and academia, and poses important challenges to traditional toxicity testing paradigms. In fact, more proper models of risk assessment of the possible dangerousness of AgNPs, when used in the productive food chain, are necessary and useful to improve the risk perception by stakeholders.

Within this context, our work aimed at studying the dissolution behaviour of AgNPs, simulating the human oral ingestion and the passage along the gastrointestinal tract. We implemented an *in vitro* test and provided a description of the bioaccessible ionic species (released free and digestive-matrix bound), by using a range of complementary analytical techniques. Moreover, we improved the work evaluating the bioavailability *in vivo* of AgNPs.

Our data indicated that dissolution of AgNPs is complete already at the stomach compartment, with 19% of released free ions and 81% matrix-bound. Passing in the intestine, the resulting ions are mostly bound to the digestive matrix in an extent close to 98% (which probably could be excreted by feces). The other 2% is bioavailable for the duodenal translocation, and could be possibly distributed, only in a low amount, through the blood circulation and the urinary tract. To confirm these preliminary observations, we extended the experimental plan with animal studies. We revealed that the major fraction of ingested silver is detected into the feces, whereas only a small portion of 2% bioavailable is found in blood and urine of mice (0.06% and 0.02%, respectively). Reasonable, the remaining bioavailable fraction (1.92%) is distributed in the tissues.

Overall, these results show a correlation between the *in vitro* outcomes and the *in vivo* adsorption. Therefore, the dissolution test may be a useful predictive analytical tool, *in vitro*, for quantitative monitoring

and risk assessment of nanoparticles with known capacity to dissolve in human oral exposure conditions. Its standardization may be at service of regulatory for quality control check in industrial nanoparticle chain.

## ASSESSING THE EFFECTIVENESS AND ENVIRONMENTAL RISK OF NANOCOPPER-BASED WOOD PRESERVATIVES

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Nanotechnology is changing our lives and provides novel solutions in all areas of material research and development. In wood protection, nanomaterials have recently been developed to outperform conventional wood preservatives and lengthen the service of life of timber structures. These nanoparticles-based wood preservatives are commonly known as “micronized copper”, which are composed of basic copper carbonate nanoparticles and a second organic biocide [1].

Copper-based nanoparticles are frequently associated with adverse health effects, however the environmental fate of micronized copper – which is already present in the market – is not clear yet [2]. In this paper we investigated whether the use of nanoparticles in micronized copper used for wood protection has an added value, we monitored, quantified and visualized the fate of copper from its state as wood preservative formulation, after impregnating wood and finally after artificial ageing of treated timber.

In particular, two possible release pathways were assessed: wood dust produced by mechanical abrasion of the treated wood, and release via sporulation by copper tolerant wood-destroying basidiomycetes.

On the basis of the experimental results, micronized copper effectively protected the wood from soilborne fungi and wood-destroying basidiomycetes, and homogeneously penetrated into easily treatable wood species. However, the treatment is not effective against copper-tolerant wood-destroying fungi [3], and refractory wood species are only poorly treatable [4]. Copper can be released into the air either via abraded wood dust particles [5] or by copper containing spores of copper-tolerant fungi. Preliminary cytotoxicity results did not reveal a specific nano-hazard, but indicate that a more detailed assessment is required which is currently being investigated.

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## NanoStreeM to assess exposure and risks of nanomaterials in nano-electronics manufacturing

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In order to maximize the benefits of nanotechnology and avoid unwanted consequences, additional data are needed to better understand potential health risks and necessary control measures across the life cycle stages of novel nanoscale or nano functionalized materials.

By comparing various risk assessment approaches and sharing good practices, the European NanoStreeM project will contribute to the improvement of the awareness and safety of workers in the semiconductor industry, and to the minimisation of the impact on the environment. The goal of this project is to gain insight in the potential pathways of exposure to and release of nanomaterials, to compile risk management practices enabling better risk governance in the European semiconductor industry, and to serve as benchmark for other industries.

The results on the evaluation of various risk assessment methodologies will be presented. These methodologies are compared for e.g. their user-friendliness, expert level requirement, and type of assessment (exposure, risk, banding, hazard,...). Quite a number of risk assessment methodologies are (being) developed for safety evaluation of engineered nanoparticles. Are models developed for chemicals in the non-nano form suitable to assess the safety of nanomaterials? The first ranking of the models/methodologies is based on their quantitative or qualitative output (Table 1). Some models that will be discussed are the Precautionary matrix, Stoffenmanager nano, Art, RiskOfDerm, Ecetoc-TRA, Sofokles, the CB Nanotool, the ISO-approach, and the near-future Guidenano-tool.

Table 1: Methodologies and tools for exposure and risk assessment

<b>Models/approaches</b>	<b>Number of identified tools/approaches</b>	<b>Examples of tools/approaches</b>
(semi) quantitative approaches	12	DNEL derivation, exposure measurements, tiered approaches, databases (ECHA, OECD)
(semi) quantitative models	12	ART, Riskofderm, DREAM, Nanosafer, Consexpo, ENPRA model, ECEL, ECETOC-TRA, Guidenano tool
qualitative approaches and	17	Mainly Control Banding tools: ESIA approach for CMP,

models		Stoffenmanager-nano, ISO-CB, CB Nanotool, Precautionary Matrix, LICARA
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The models are evaluated for their applicability in the semi-conductor industry, if need be after model adaptation, by testing them in real-life exposure scenarios. To fill gaps in the exposure assessment we will make recommendations for use of exposure monitoring complementary to risk and control banding in the semiconductor industry. The findings will lead to a guidance on the use of risk assessment methodologies for the semi-conductor industry. It is quite a challenge to write a guidance that is also be applicable for future nanomaterials and processes.

*The NanoStreeM project (Nanomaterials: strategies for safety assessments in advanced integrated circuits manufacturing) receives funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement n° 688194.*

## NANOMATERIAL COMPUTATIONS IN YOUR BROWSER: PREDICTIONS, READ-ACROSS, EXPERIMENTAL DESIGN & INTERLABORATORY COMPARISON USING THE JAQPOT MODELING PLATFORM

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In the field of nanomaterial safety assessment, there are many tools that offer users useful aid for their modeling needs. Apart from possible financial barriers and restrictive licenses, users often have to work through the various file formats, technical and scientific proficiency needed, lack of visibility into tool operation and often limited interoperability and reproducibility are all factors that have hindered a broader use of modeling tools. With the goal to satisfy the need for harmonisation in terms of databases, ontology and modelling infrastructures, Jaqpot Quattro<sup>1</sup>, an open source web application for nanomaterial modeling was developed within the eNanoMapper FP7 project<sup>2</sup>, providing a user-friendly interface for nanoQSAR modelling, validation services, read across predictions, optimal experimental design and inter-laboratory testing. Furthermore, Jaqpot Quattro performs modelling, data preprocessing procedures, while providing APIs for dynamic algorithm integration, integration with third-party services and algorithm implementations. Users can view and improve its code, receive guidance from tutorials on the user interface<sup>3</sup>, APIs<sup>4</sup> and modelling services<sup>5</sup> and build their own services following the OpenTox-compliant API<sup>6</sup>.

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4. <http://www.enanomapper.net/library/modelling-services> Video Tutorial
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6. Accessible through the Swagger instance of the API at: <http://jaqpot.org:8080/jaqpot/swagger/>

## NANO-SPECIFIC RISK MANAGEMENT MEASURES (RMM) AND A PROPOSAL FOR EFFECTIVENESS FACTORS FOR MODELING PURPOSES

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Exposure to nanoparticles is associated with a wide range of industries, types of nanomaterials, potential sources of exposure and control strategies. The relevance of different engineering and PPE controls will be dependent on the life cycle stage where the nanomaterial is used, incl. the industry and scenario of concern. Our aim was to collate available information on the efficiency of Risk Management Measures (RMM) focusing on workplace control measures that are relevant for activities or processes associated with nanomaterials. Where required, evidence on the effectiveness of control measures relevant for 'conventional' substances was also considered and integrated with nano-specific data. In order to propose an effectiveness value, the different groups of control measures were analysed to determine if there are any differences in the effectiveness of control measures for nano-sized aerosols and larger particles.

For this purpose, a few research questions include:

- (1) Are there any differences in the effectiveness of control measures for nano- versus non-nano materials? If so, which factors determine these differences?
- (2) Can existing evidence of conventional substances be replaced, refined or extrapolated with new nano-specific data?
- (3) If nano-specific data is not available, can existing evidence on control measure effectiveness (relevant for 'conventional' substances) be applied or adopted for nano-specific scenarios?

Based on a literature review conducted in 2014, available peer reviewed papers and other existing data were entered in an adapted nano-specific module of ECEL (named nano-ECEL). The studies found were cross-checked with more recent reviews on the topic and supplemented with additional data. Besides peer reviewed data, grey literature and experimental studies within various EU projects were included in the review and analysis.

Three main groups of RMM were identified: engineering controls, respiratory protective equipment (RPE) and skin protective equipment (SPE). In total 30 studies were found that met the inclusion criteria from which a quantitative effectiveness value could be derived. For all RMM groups limited data is available to evaluate their effectiveness specifically for nano. Numerous studies were found that are relevant for a qualitative evaluation of RMM related to nanomaterials, and were included in the evaluation process.

This information was used to determine whether existing effectiveness values of RMM for conventional substances require updating for nano-specific scenarios, and if so, the rationale for adapted values and the available evidence and data available to underpin these values. This effectiveness classification can be used for exposure and risk modelling purposes.

## A High-Throughput Screening Approach Evaluated Toxicity of 31 Engineered Nanomaterials Generated for the NANOSOLUTIONS Project

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Accurate, predictive and rapid *in vitro* toxicity assessments are needed to accompany and eventually replace the expensive and slow traditional *in vivo* toxicity test of engineered nanomaterials (ENMs). Under this aim, we developed a multi-readout *in vitro* high-throughput screening concept to evaluate and rank ENM toxicity. We applied the human lung epithelial BEAS-2B cell line at the 384-well format with or without 10% fetal bovine serum. Assaying without serum allowed minimization of ENM corona effects whereas assays with serum allowed for comparability to models routinely grown with serum. Early testing showed similar growth characteristics of the BEAS-2B model with or without serum, and thus permitted this testing strategy. Applying both luminescence and fluorescence based assays, the cells were exposed to the NANOSOLUTIONS ENM collection and a panel of chemical controls at 8 concentrations for 6, 24 and 72 hours. Cellular ATP content, cell number changes, apoptosis, and antibody-based detection of DNA damage and oxidative stress induced RNA/DNA damage was assessed, and ENM-mediated assay interference was corrected for in each assay. Encompassing >110 000 separate analyses, the NANOSOLUTIONS NMs demonstrated manifold differences in toxic potency, including related to coating with NH<sub>2</sub>, COOH or PEG. As expected, lower toxicities were generally, although not consistently, seen in the presence of serum. Silver-NH<sub>2</sub> caused higher toxicity than Ag-COOH and Ag-PEG. Silver-NH<sub>2</sub> also induced DNA and RNA damage. CdTe Quantum Dots coated with NH<sub>2</sub>, COOH and PEG were highly toxic, and moreover, induced DNA and RNA damage and apoptosis in the absence of serum. Gold ENMs, including gold-NH<sub>2</sub> at size of 5 and 20 nm, were toxic, whereas Au-PEG-20nm was totally inert. Overall, assessment of an array of endpoints, concentrations and exposure times efficiently demonstrated influences of the selected ENM surface modifications. High content analysis-based measurement of the more specific DNA and RNA damage or apoptosis endpoints served to validate the viability assays, and provided further mechanistic insight. We conclude that our approach for multi-readout HTS-mediated toxicity evaluation is able to generate initial toxicity potency screening data within days, and thereafter the approach enables deeper and iterated evaluation of toxicity mechanism within weeks. The results argue collectively for considering a proactive HTS technology-mediated safe innovation strategy for novel ENMs generation.

## **GUIDEnano: A web-based tool for risk assessment of nanomaterials and nano-enabled products**

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Within the EU FP7 project GUIDEnano, a web-based guidance tool has been developed to support industry in the evaluation and management of human and environmental health risks of nano-enabled products. The modular structure of the tool allows the user to define the scope of each risk assessment, which can range from a specific exposure scenario to the entire product life cycle, and that can consider both human and environmental risks. The core of the tool is organized in the following modules: material characterization, life cycle activity description, fate, exposure, toxicity, and risk assessment. The information provided by the user and derived from the incorporated data and knowledge, flows throughout these modules to provide a quantitative risk assessment for each scenario of interest. Depending on the outcome of the risk assessment, a range of risk mitigation measures is offered to the user for consideration.

During the GUIDEnano project, efforts have been focused on structuring and integrating knowledge and information made available by the different partners to be incorporated into the tool, as well as on generating experimental data to fill in critical data in the risk assessment process. As a result, the GUIDEnano tool combines a range of predictive models, multilevel decision trees, and databases to derive critical information along the risk assessment process.

The user is required to fill in case specific information, at least regarding the materials/products characterization, the scale of the assessment (in terms of time span and amount of materials involved), and the activities and exposure scenarios under consideration. When expertise and availability of data allows it, the user has the option to introduce much more case-specific characterization data. For example, in terms of geometric and chemical composition of the local environmental compartments, or in terms of toxicity studies. In addition, during the whole process, flexibility is provided to the user to introduce data specific estimates derived from in-house data (e.g., exposure assessment field campaigns), identified in the literature, or obtained in specialized predictive models outside the GUIDEnano Tool.

The knowledge has been modelled using Intelligent Objects™ and follows an object oriented approach that allows modular development and easy updating. As far as possible, the risk assessment process has been designed following widely accepted assumptions, keeping in mind the current regulatory framework for chemicals in Europe (REACH), and sometimes moving beyond to what the consortium expects will be upcoming regulatory updates in relation to nanotechnology. The structure of the tool will allow updating it to accommodate potential extensions identified in the project (such as semi-probabilistic assessment), and achieved in future nano safety research projects.

Sustainability of the tool after the GUIDEnano project is a high priority, and a corresponding business plan is being finalized to continue further development and exploitation of the GUIDEnano tool.

# **SUNDS, A MULTI-CRITERIA BASED DECISION SUPPORT SYSTEM FOR NANOTECHNOLOGY RISK ASSESSMENT & MANAGEMENT**

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The SUN Decision Support system (SUNDS) web application software has been developed in the SUN European project on Sustainable Nanotechnologies ([www.sun-fp7.eu](http://www.sun-fp7.eu)). The software aims at supporting decisions on assessment & management of nanomaterials and nano-enabled products in industry, regulatory bodies and insurance companies.

The proposed sustainability assessment applies a two tiers approach which, on the basis of the supplied information, is able to generate qualitative or quantitative results (1). Moreover, a certification standard questionnaire is present which is based on the CENARIOS® certification standard by TÜV SÜD Industrie Service GmbH (2).

The first assessment tier is based on the Licara Nanoscan method which supports SMEs in assessing benefits and risks associated with new or existing nanoproducts (3). The second assessment tier is based on an adaptation of the authorisation process currently in operation within the EU REACH regulation. REACH is based on Risk control (RC), demonstrating adequate control of risk due to a substance's use, and Socio-economic Assessment (SEA), demonstrating that benefits of using the substance significantly outweigh societal costs. SEA analyses are based on the triple bottom line approach, which comprises the environmental, economic, and societal 'pillars'.

The methodology developed in the software concerns the assessment of different sustainability aspects through the calculation of qualitative and quantitative results from the application of state of the art assessment methodologies for RC and SEA. To face the substantial heterogeneity of information a Multi Attribute Value Theory specific assessment methodology is proposed which is intended to capture inputs relations and aggregate results by means of stakeholders' insights.

The web application software has been mainly programmed in the ECMAScript 2016 programming language by the use of the Meteor framework. The underlying architecture is composed by a Node.js server equipped with a MongoDB No-Sql database. Results are presented in a user friendly graphical user interface which makes widely use of dynamic charts provided by the Highcharts framework.

The software has been tested by the application to case studies including nano-copper oxide -based biocidal paint and plastic car bumper coloured with nano-organic pigment.

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## DECISION SUPPORT IN COLLECTIVE DIALOGUE ON INTERNATIONAL REGULATION OF NANOMATERIALS

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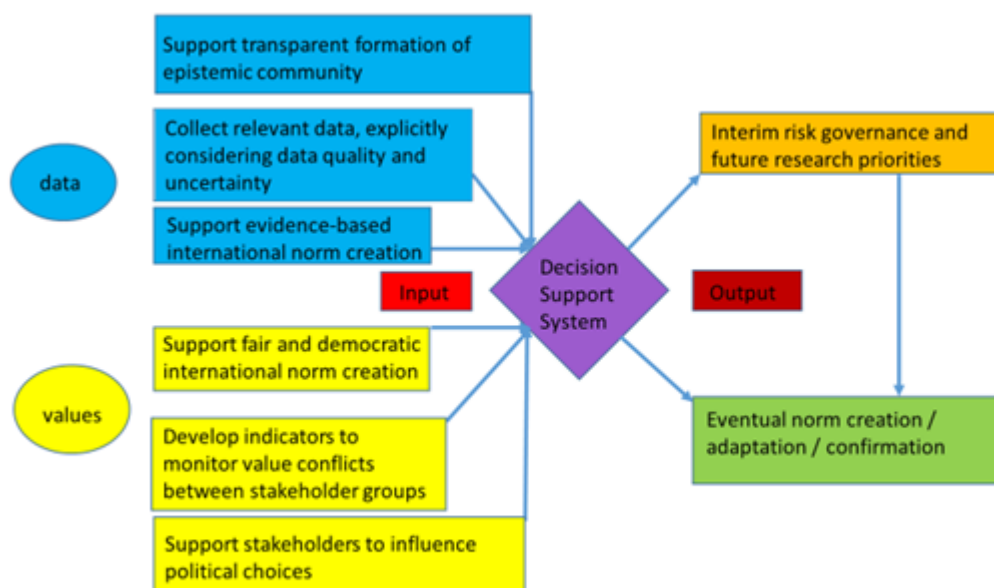
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In the project on Sustainable Nanotechnologies ([www.sun-fp7.eu](http://www.sun-fp7.eu)), we developed a software decision support system (SUNDS), aimed at supporting decisions on safe and sustainable management of nanomaterials and nano-enabled products in industry, regulatory bodies and insurance companies. In order to target the needs of the relevant decision makers, we started by soliciting their needs through a survey [1], semi-structured interviews [2] and three stakeholder workshops [3]. The findings inspired the design of the SUNDS tool. In addition, the responses were analysed based on an adapted mental modelling approach. The latter has evolved into a more philosophical reflection on possible future roles decision support might play in international governance of nanomaterials, in particular through offering separate input channels for science-based data and subjective values, allowing decision makers to consider both aspects simultaneously (Figure 1). In this presentation, we will present our main findings of the stakeholder engagement in the SUN project, in particular during the third workshop in Edinburgh, on 6 October 2016. We will also sketch some options for future follow-up projects.

Figure 1: overview of aims of data and value inputs in the online decision support system for nanomaterials



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**#1553**

**CHRONIC ECOTOXICITY OF COPPER OXIDE NANOMATERIALS TO THE POND SNAIL *LYMNAEA STAGNALIS***

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Nanotechnology is a rapidly developing field and the commercial use of nanomaterials for novel applications continues to increase. Copper oxide nanoparticles (CuO NPs) are frequently employed for their antimicrobial properties in antifouling paints and other applications. Their extensive use can lead to contamination of aquatic ecosystems.

The main objective of this study was to investigate the chronic sublethal toxicity of different CuO NPs to different life stages of the great pond snail *Lymnaea stagnalis*, a representative organism of the benthic ecosystem. It has become increasingly recognised that sublethal effects as measures of toxicity, are far more sensitive in the assessment of the potential impact of chemicals within an ecosystem.

Chronic sublethal concentrations were selected based on the data gathered from acute exposure studies. Experiments aimed to investigate the effects on the reproduction and growth of *L. stagnalis* following exposure to Cu, either as ionic Cu (CuSO<sub>4</sub>), pristine CuO NPs or “safe by design” CuO NPs. The latter were CuO-ASC and CuO-PVP NPs with, respectively, Na ascorbate and Polyvinylpyrrolidone as capping agents. Young adult snails (22±2mm) were exposed to Cu materials at 20°C for 30 days in a semi-static experiment. Endpoints such as: mortality, feeding rate and weight changes were also evaluated, along with the reproduction parameters.

LC50<sub>30d</sub> values estimated for exposure to Cu as CuSO<sub>4</sub> and pristine CuO NPs were, respectively, 60 µg L<sup>-1</sup> Cu and 500 µg L<sup>-1</sup> Cu, indicating greater toxicity of the ionic form. Lethal effects were not investigated for the modified CuO, where snails were only exposed to sublethal concentrations.

Results from the reproduction endpoints are in accordance with the lethality trend. EC50<sub>30d</sub> values estimated for the fecundity endpoints were 4 fold lower for the Cu as CuSO<sub>4</sub> than pristine CuO NPs.

Additionally, exposure to “safe by design” CuO NPs showed significant effects (One-way ANOVA, p<0.001) on the growth and reproduction parameters relative to the control; indicating a higher toxicity of CuO-ASC in the long term.

The experiments' results demonstrate a time-related increasing toxicity of CuO NPs to *L. stagnalis*, emphasizing the need for more chronic studies to accurately evaluate the impact of nanomaterials in the real environment.

Furthermore, long-term experiments using juveniles *L. stagnalis* exposed to CuO NPs are ongoing, evaluating growth and time-related expression profiles of antioxidant enzymes and heat shock proteins response in snails to thermal shock.

This research project is funded by the European FP7 project SUN “Sustainable Nanotechnologies”.

## **SOLUTIONS TO PRACTICAL CHALLENGES IN DEVELOPING DISPERSION PROCEDURES FOR NANOPARTICLE CHARACTERIZATION AND TOXICOLOGICAL TESTING**

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In large-scale scientific projects where nanomaterials need to be investigated by a number of research groups with different scientific background it is necessary to assure that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. One major challenge is the preparation of stock dispersions from nanomaterials provided as powders as distinct dispersion procedures may introduce variability in the toxicity or characteristics that are measured. Stock dispersions are used in a variety of toxicological tests where aliquots of the stock suspension are typically added to the relevant test medium, e.g. cell culture medium. Furthermore, stock dispersions are required for particle characterization, as many techniques, like dynamic light scattering, laser diffraction, analytical ultracentrifugation, nanoparticle tracking analysis, are only able to measure aqueous samples. In order to obtain meaningful results and to allow cross-comparison of different toxicity and characterization tests and assays, it is therefore crucial to develop efficient and reproducible dispersion procedures. These harmonized and standardized protocols have not only to be efficient, but also be feasible in the majority of test laboratories. Common limitations include the availability of dispersion equipment in the involved laboratories and the access to analytical equipment for characterizing and checking the quality of the dispersions. Further a compromise has to be found regarding, the (maximum) concentration of the stock dispersion, the resulting stock dispersion volume, and the composition of the dispersion medium, because of the variety of (eco)toxicology tests with each having specific requirements. The presentation will summarize the major challenges and the corresponding solutions of the NANOSOLUTIONS project with regards to stock dispersion preparation. As a specific example the development of a common dispersion procedure for copper oxide nanoparticles with different surface functionalization (ammonium, carboxylate, or polyethylene glycol) will be presented. For this nanomaterial, a dispersion SOP was developed which included a calorimetric method for calibration of the delivered acoustic energy by adjustment of the probe-sonicator amplitude. Additionally, an SOP was established that described the conduction of dynamic light scattering (DLS) measurements for determination of hydrodynamic size and size-distribution of the nanoparticles in the final stock dispersion. The SOPs were tested by ten laboratories. In most cases deviations of the determined sizes could be explained with deviations from the procedure described in the SOP. The performed work showed that it is possible to obtain comparable stock dispersions in different laboratories if carefully prepared SOPs are provided which consider the most important parameters that influence the dispersion process and the following characterization step.

Acknowledgements: The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 309329.

***On the Fast Track towards in vitro screening of manufactured nanomaterials***

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The NanoMILE Project aims to establish a fundamental understanding of the mechanisms of nanomaterial interactions with living systems and the environment across the entire life cycle of nanomaterials and in a wide range of target species. Due to the diversity of manufactured nanomaterials (MNM) regarding chemical composition, surface modification, size, and other parameters the number of materials to be tested for safe application is steadily increasing. For effective safety screening of numerous MNMs it is necessary to speed up the testing by using *in vitro* test systems and applying High Throughput Screening (HTS)/ High Content Analysis (HCA) methods.

At the HTS facility of the EURL ECVAM laboratory of European Commission Joint Research Centre we have established a screening platform based on High Throughput Screening /High Content Analysis techniques to screen for the most relevant MNMs and endpoints using both classical and novel biomarkers. The materials tested were selected from a library of different MNMs of different sizes, different chemistry and coatings/modifications including aged, ultrasmall and also micron sized particles for comparison. The particles were characterized both as pristine particles and as in test conditions. To date, more than 100 MNMs from the NanoMILE library have been screened at the EURL ECVAM HTS facility. Main findings of this screening will be discussed.

Since the liver is a major target organ for MNM toxicity, a human hepatoma cell line, HepaRG, was used as *in vitro* cell model. The selected endpoints were based on knowledge acquired from Adverse Outcome Pathways (AOPs), a conceptual construct that describes existing knowledge on the link between a molecular initiating event and an adverse outcome. With the multi-parameter HTS/HCA assays, which are performed by fully automated robotic procedures, we contemporaneously assessed total cell number, nuclear size and intensity, mitochondrial membrane potential and cell viability as well as steatosis and apoptosis together with the nuclear parameters.

We have analysed also cell population responses and show that such an approach increases the reliability and robustness of toxicity data derived from conventional cell-based assays and provides more insight into the possible underlying mechanisms of cytotoxicity and cell death related to *in vitro* cellular exposure to nanomaterials. Examples of analysis of cell population responses of the human hepatoma HepaRG cell line treated with low-solubility oxide nanomaterials will be demonstrated.

Hazard ranking of MNMs should be a tiered approach, where High Throughput Screening/High Content Analysis provides us with an excellent tool to obtain rapidly important amount of reliable and reproducible data.

**#1644**

**Cytotoxicity screening of panel of 31 nanomaterials in the human monocytic cell line THP.1 versus primary human monocyte-derived macrophages: assessing the role of surface modifications**

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## GENOTOXICITY OF 31 NANOMATERIALS IN HUMAN BRONCHIAL EPITHELIAL CELLS

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The possible carcinogenicity of engineered nanomaterials is one of the key questions in evaluating their hazard to human health. In vitro assays for genotoxicity are used to detect carcinogens that act through a primary genotoxic mechanism. We have assessed the genotoxicity of 31 nanomaterials in human bronchial epithelial BEAS-2B cells using the alkaline comet assay for the detection of primary DNA damage (24-h treatment) and the cytokinesis-block micronucleus assay (48-h treatment) to reveal chromosome damage. The nanomaterials tested included CuO nanoparticles (NPs), two sizes (3.5 nm and 20 nm) of gold NPs, TiO<sub>2</sub> NPs and nanorods, silver NPs, quantum dots, nanodiamonds, and multiwalled carbon nanotubes (MWCNTs). All materials were studied in three different forms – with amino-, carboxyl- and polyethylene glycol- (PEG) functionalization. An uncoated form was also available for CuO, TiO<sub>2</sub>, and MWCNTs.

Dose selection for the genotoxicity assays was based on cytotoxicity assessment. Quantum dots were highly cytotoxic regardless of functionalization, suggesting that the effect was driven by the chemical composition of the nanomaterial. All forms of MWCNTs were non-cytotoxic. For the other nanomaterials except nanodiamonds, amino-functionalization strongly increased cytotoxicity. Cytotoxicity was enhanced by carboxyl-functionalization in the case of CuO and by PEGylation in the case of TiO<sub>2</sub> NPs and Ag NPs. Uncoated CuO NPs were also cytotoxic, unlike uncoated TiO<sub>2</sub> NPs and rods.

Uncoated and amino-functionalized CuO NPs induced micronuclei but no DNA damage. The 3.5-nm gold NPs induced DNA damage with amino- and PEG-functionalization and micronuclei with amino-functionalization. The 20-nm gold NPs produced DNA damage when amino-coated and micronuclei when PEG-coated. The uncoated and PEGylated forms of TiO<sub>2</sub> NPs increased micronuclei, and an equivocal elevation of DNA damage was seen for the uncoated and amino-functionalized forms. Uncoated TiO<sub>2</sub> nanorods produced DNA damage. For silver NPs, the PEGylated form increased DNA damage and micronuclei and the carboxylated form micronuclei. Only quantum dots were able to induce micronuclei with every surface functionalisation. Amino-functionalized quantum dots also increased DNA damage. Uncoated MWCNTs were not genotoxic, but carboxylated and amino-functionalized MWCNTs produced an equivocal result in the micronucleus and the comet assay, respectively. Nanodiamonds were not genotoxic.

In conclusion, DNA damage was increased by amino-functionalized quantum dots, PEGylated silver NPs, amino-functionalized 20-nm gold NPs, amino- and PEG-functionalized 3.5-nm gold NPs, and uncoated TiO<sub>2</sub> nanorods, with an equivocal result for uncoated and amino-functionalized TiO<sub>2</sub> NPs and amino-functionalized MWCNTs. Micronuclei were induced by all functionalized quantum dots, PEGylated and carboxylated silver NPs, amino-functionalized 3.5-nm gold NPs, PEGylated 20-nm gold NPs, and uncoated TiO<sub>2</sub> NPs, with an equivocal effect by carboxylated MWCNTs. Although genotoxicity was seen more often with the amino-coated (and PEGylated) forms than with the carboxylated forms, general rules could not be derived for the

effects of functionalisation. The fact that the amino-functionalized nanomaterials tended to be very cytotoxic may have partly masked their possible genotoxic effects.

[Supported by NMP4-LA-2013-309329 NANOSOLUTIONS]



***In vivo* genotoxic and inflammatory effects of a self-cleaning agent containing silver-doped TiO<sub>2</sub> nanoparticles**

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The GUIDEnano project, funded under the 7th framework programme (Grant Agreement No.604387), aims at developing a web-based guidance tool, which will help to evaluate the risks of nano-enabled products on human and environmental health throughout their life cycle.

GUIDEnano Tool v2 was recently released and it is validated by case studies in co-operation with industrial partners. In the present case study, a self-cleaning agent containing titanium dioxide (TiO<sub>2</sub>) nanoparticles (NPs) was tested *in vivo* for pulmonary and systemic genotoxicity and inflammatory effects in mice.

Due to the photocatalytic and oxidative properties, TiO<sub>2</sub> NPs can be used to create dirt-repelling and antimicrobial coatings. The self-cleaning agent tested is applied on surfaces as an aerosol by spraying and can thus be inhaled by workers and consumers during the application process. Previous studies suggest that TiO<sub>2</sub> NPs can cause acute pulmonary inflammation and act as an airway irritant. However, the genotoxicity of TiO<sub>2</sub> NPs remains controversial and further studies are required.

In the present study, a water-based self-cleaning agent containing ~0.5 wt% nanoTiO<sub>2</sub> (particle size <8 nm, doped with silver (Ag)) was administrated to C57Bl/6 mice by repeated (3x) pharyngeal aspiration at three different doses (corresponding to 10, 30 and 80 µg of TiO<sub>2</sub>/mouse/aspiration). 1 and 28 days after the last administration, DNA damage was assessed by the Comet assay locally in bronchoalveolar lavage (BAL) and lung cells, and systemically in liver cells. Micronuclei, a biomarker of chromosome damage, were analysed in bone marrow and peripheral blood erythrocytes. Immunotoxicity was evaluated by BAL cell counting. Furthermore, histopathological effects on the lungs are being assessed. To differentiate the possible effect of the Ag doping, exposure of the highest tested dose of the self-cleaning agent (3 x 80 µg of TiO<sub>2</sub>/mouse/aspiration) was repeated with a dispersion of undoped TiO<sub>2</sub>.

At 24-h, a mild, statistically significant increase in DNA damage was seen at the lowest tested dose in liver cells. 28 days after the last administration, a slight statistically significant increase in DNA damage was measured in the BAL cells. No systemic genotoxic effects were observed in bone marrow at 24 h or in blood erythrocytes at 28 d.

The self-cleaning agent tested induced an activation of inflammatory response. A dose-dependent recruitment of neutrophils, accompanied with mild influx of eosinophils and lymphocytes was detected by BAL cell counting 24 h after the last administration. 28 d later, no significant increases in the numbers of neutrophils or eosinophils was seen in BAL fluid, indicating that the inflammation had resolved.

Our findings show that short-term exposure of mice to nanoTiO<sub>2</sub>-containing self-cleaning agent induces pulmonary inflammation and slight DNA damage but no systemic genotoxic effects.

## A 3D HUMAN CO-CULTURE MICROTISSUE MODEL FOR NANOPARTICLE EFFECT AND UPTAKE STUDIES AT THE PLACENTAL BARRIER

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Theme: Hazard assessment along the life cycle of nano-enabled products

The placenta is an organ responsible for the growth, development and the protection of the fetus against various xenobiotics. With the growing use of nanotechnology, the placenta is likely to come into contact with nanoparticles (NPs) either accidentally through exposure or intentionally in the case of nanomedical applications. In both cases, it is of major importance to better understand NP effects and uptake mechanisms at the human placental barrier. However, knowledge on NP-placenta interaction is limited and concepts how to steer barrier translocation are essentially lacking.

We developed a human 3D placental *in vitro* microtissue (MT) model using the scaffold-free hanging drop technology. Morphological analysis revealed the formation of well-organized MTs consisting of a core of placental fibroblasts surrounded by a trophoblastic choriocarcinoma cell layer (BeWo cells), resembling the *in vivo* placental barrier structure. Trophoblasts exhibited a polarized morphology with extensive apical microvilli and formed tight junctional complexes. BeWo cells secret more of the placental hormone human chorionic gonadotropin (hCG) if cultivated in co-culture MTs as compared to 2D monocultures indicating that differentiation of BeWo cells on 3D MTs is enhanced. Cell viability studies showed that CdTe and CuO NPs but not TiO<sub>2</sub> induced a concentration-dependent decrease in MT viability. In addition, CdTe and CuO NPs significantly reduced hCG levels at low concentrations. Therefore, exposure to CuO and CdTe NPs during pregnancy should be avoided.

The newly developed human placental MT model is expected to allow the pre-screening of different NPs in terms of acute toxicity which are indispensable for the safe development of NPs for industrial, commercial and medical applications. In addition to acute toxicity assessment, the co-culture MT model holds great potential for the assessment of NP penetration and uptake mechanisms because a more tissue-like transport of NPs can be expected due to the 3D structure. Finally, we anticipate that the inclusion of additional cell types such as immune cells or endothelial cells as well as the replacement of BeWo cells with primary trophoblasts will further improve the predictive value of this promising new 3D placental microtissue model.

*This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 309329 (NANOSOLUTIONS) and no. 263215 (MARINA).*

# THE ENDOTHELIAL GLYCOCALYX CONTROLS INTERACTIONS OF ENGINEERED NANOMATERIALS WITH THE VESSEL WALL AND THEIR TRANSLOCATION ACROSS THE BLOOD-TISSUE BORDER

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The endothelial glycocalyx (eGCX) is a negatively charged meshwork of proteoglycans, glycoproteins, glycosaminoglycans, and plasma proteins that, in addition to its role in vascular homeostasis, provides a physical barrier against the endothelial adhesion of particulate blood constituents, such as platelets and leukocytes. The eGCX can be perturbed or even shed under many pathological conditions, including inflammation, atherosclerosis, and ischemia-reperfusion injury. Here, we addressed the question of whether an intact eGCX might also represent a barrier against the uptake of engineered nanomaterials (ENM) by endothelial cells and their translocation across the blood-tissue border.

In a first set of *in vitro* experiments, the expression of the eGCX on cultured human umbilical vein endothelial cells (HUVECs) was determined by immunostaining of heparan sulfate. Enzymatic degradation of the eGCX was achieved by incubating the cells with eGCX-shedding enzymes and the uptake of 50-nm polystyrene nanospheres was quantified by confocal microscopy. HUVECs expressed a robust eGCX when cultured for 10 days. Moreover, the uptake of both carboxyl- and amine-functionalized polystyrene nanospheres was significantly increased in cells with a degraded glycocalyx, while it remained at a low level in cells with an intact glycocalyx. In a second set of *in vivo* experiments in a murine model, glycosaminoglycans of the eGCX were found to physically cover endothelial adhesion and signalling molecules thereby preventing endothelial attachment, uptake, and translocation of carboxyl-functionalized quantum dots through different layers of the vessel wall. Conversely, degradation of the endothelial glycocalyx under pathologic conditions (ischemia-reperfusion) promoted interactions of these ENM with microvascular endothelial cells.

In summary, we demonstrate (i) that the eGCX constitutes a barrier against the internalization of blood-borne nanoparticles by endothelial cells, (ii) that glycosaminoglycans of the eGCX physically cover endothelial adhesion and signalling molecules thereby preventing the endothelial attachment, uptake, and translocation of ENM through the vessel wall, and (iii) that injury of the eGCX enables interactions of ENM with the microvascular endothelium under pathologic conditions. In conclusion, these data suggest that the eGCX might play a crucial role for interactions of blood-borne ENM with the vessel wall and their translocation to extravascular tissue, a finding with far-reaching implications for the fields of nanotoxicology and nanomedicine.

## Development of an *in vitro* method to study long-term effects of copper nanoparticles on fish cell lines

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Copper oxide nanoparticles (CuONPs) are widely applied and their increasing production has led to major concerns regarding the potential risk of CuONPs release from consumer products into aquatic environments. Although CuONPs have been described as toxic for aquatic organisms in a number of acute studies there is a general lack of knowledge about their long term toxic effects. The objective of this work is the development of an *in vitro* method to explore the cytotoxic effects of CuONPs on fish cell lines after long-term, low-dose exposures, simulating a more realistic environmental scenario.

The tool was tested in two fish cell lines: RTL-W1, originated from a rainbow trout normal liver and CLC, a carp leukocyte cell line. Both cell lines were exposed for 21 days to low CuONPs concentrations, taking as reference those concentrations used in short-term (24 hours) experiments that were most close to the 24 h EC50 and EC10 values. Cells were also treated with CuSO<sub>4</sub> salt as a control source of ions. Cytotoxicity was assessed at 1, 7, 14 and 21 days of CuONPs exposure by three different endpoints (alarmarBlue, CFDA-AM and NR assays) that discriminate among different mechanisms of cellular toxicity (metabolism activity, plasma membrane integrity and lysosomal functionality, respectively). Transmission electron microscopy (TEM) analyses of cells exposed to the same CuONPs concentrations were carried out at different times of treatment in order to explore the subcellular mechanism underlying CuONPs toxicity. A different behaviour in the face of CuONPs and CuSO<sub>4</sub> exposure was observed comparing the two cell lines: CLC were more sensitive to copper nanomaterial and salt, reaching 100% of mortality after 7 days of treatment with 50µg/mL (24h EC10) of CuONPs, and 20-50% (depending on the assay) of mortality at only 0.5µg/ml at day 21. By contrast, RTL-W1 viability was not affected after exposure to 25 µg/ml (24h EC10) CuONPs for 21 days, but 100 µg/ml (24h EC50) caused approximately 90% mortality at this time. TEM images of CLC cells treated with 5µg/mL of CuONPs for 7 days showed a granulated material inside vacuolated structures within the cytoplasm that might be related with a degradation-dissolution mechanism of the nanomaterial by the cell. Also, some granulated material compatible with CuONPs was observed inside the cell nucleus. CLC treated with higher CuONPs concentrations for longer periods of time were severely affected.

The *in vitro* protocol described here is a versatile tool to study the cytotoxic effects of continuous low-dose exposure to different nanomaterials (not only CuONPs) in a more realistic scenario for prediction of their toxicity in *in vivo* bioaccumulation studies and for the support of the 3Rs initiative in the development of alternative ecotoxicological tests.

Acknowledgements - This research was supported by FP7-SUN project (Grant Agreement No. 604305).

## NOVEL MODELS AND APPROACHES FOR ASSESSING NANOPARTICLE EXPOSURE AND EFFECTS IN FISH

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Nanomaterials are used in a diverse range of products and most will enter the aquatic environment posing a potential health threat to aquatic organisms. There is evidence to suggest that some nanoparticles can induce harmful effects in wildlife but generally only at very high exposure levels. Exceptions to this are for some silver based nanomaterials that have been shown to be biologically active for environmentally relevant exposures. Fish act as sentinels for water quality and form part of a suite of aquatic animals used in OECD guideline tests. It is uncertain however whether current testing systems are appropriate for quantifying exposure effects of nanomaterials in fish. Here we report on the development and application of a range of analytical techniques and novel models for tracing the uptake and biological effects of nanomaterials in fish. We first consider the range of imaging methods available for tracing nanomaterial uptake into fish, focusing predominantly on anti-Stokes Raman scattering (CARS) microscopy and illustrating the localisation of nanomaterials, spanning metals to polymers, in live zebrafish. We then illustrate the application of whole mount *in situ* hybridisation in zebrafish larvae for identifying localised responses to nanomaterials through known effect pathways. Using silver nanoparticles as an illustrative example, we show responses in a wide range of body tissues for a suite of molecular markers for oxidative stress and various detoxifying enzymes (including *GSTp*, *Mt2* and *Hmox1*). Most studies to date have conducted relatively short term exposures to nanomaterials and at high exposure levels, which does not represent real life chronic exposure scenarios. High exposure levels are adopted, in part, because of the challenges in quantifying nanomaterial uptake into body tissues for low level exposures. The use of isotopically labelled nanomaterials can overcome this difficulty. Next we present an exposure of zebrafish over two generations to isotopically labelled nanosilver at environmentally relevant exposures to investigate body tissue uptake, biomarker responses and health effects outcomes, including on reproduction. In some of this work we show an enhancement in response to silver in offspring whose parents were exposed to silver nanomaterials, thus identifying new challenges for hazard assessment of nanomaterials. Early life stages in fish may be especially vulnerable to the effects of nanomaterial exposure and to investigate this we have adopted a new fish model, the live bearing, *Xenotoca eisinii*. We will illustrate its application for exposures to silver based nanomaterials. Lastly, we report on the development of a novel transgenic biosensor zebrafish model for oxidative stress (a common mechanism for nanoparticle effects) that incorporates an electrophile response elements (EpRE) sequence to drive the expression of a reporter protein that can be detected by fluorescence microscopy in live fish in real time. We demonstrate this transgenic line responds to a variety of oxidative stressors, in dose-dependent and tissue-specific manners illustrating considerable promise for studies into the oxidative potential of nanomaterials.

**#1666**

**Novel tools for hazard on ecosystems services and ecological risk  
assessment of NMs**

Janeck Scott-Fordsman

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## LOOKING INTO A PATTERN BETWEEN NANOPARTICLE COATINGS AND TOXICITY IN AQUATIC ORGANISMS

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Functionalization of manufactured nanoparticles (NPs) using different coatings involves the modification of their physicochemical and toxicological properties. This implies that complete toxicity profiles could only be established by performing a high number of eco/toxicity assays comprising all possible coating-NPs combinations. In order to cover this lack of information and to reduce *in vivo* assays, one of the objectives of the Project GUIDEnano is to establish relationships between different types of coatings of metal NPs and their associated toxicities in an attempt to establish read across approaches.

In this study we investigated how two hydrophilic coatings, citrate (CIT) and polyethylene-glycol (PEG), and two hydrophobic coatings, dodecylphosphonic acid (DDPA) and oleylamine (HPB), modulate the toxicity of the corresponding uncoated NPs of TiO<sub>2</sub>, CeO<sub>2</sub> and AgNPs, provided by PlasmaChem GmbH (Germany). Acute toxicity was studied in *Daphnia magna* following the OECD TG202. *In vitro* toxicity assays to fish were assessed in two-different fish hepatoma cell lines (PLHC-1 and RTH-149). Cytotoxicity assays (Alamar Blue, CFDA-AM, Neutral Red uptake) were conducted after an exposure period of 24 h. In addition, *in vivo* acute toxicity studies were conducted in rainbow trout following the OECD TG203. Pristine NPs and NPs in the exposure media were characterized by TEM, DLS and ICP-MS.

The four TiO<sub>2</sub>NPs were not toxic to either of the two cell lines even at 100 mg/L. CeO<sub>2</sub>NPs were neither toxic for the PLHC-1. However, the RTH-149 cell line was more sensitive showing a decrease in viability after exposure to CeO<sub>2</sub>-UNC and CeO<sub>2</sub>-CIT. The other two coated CeO<sub>2</sub>NPs did not trigger any toxicity. The higher sensitivity of RTH-149 cells was also visible after their exposure to AgNPs. Only Ag-HPB provoked cytotoxicity in PLHC-1 cells, whereas all AgNPs led to a decrease in viability in RTH-149 cells. A general pattern of toxic behavior could not be established for these coatings in the NPs tested in these fish cell lines.

The uncoated NPs produced toxicity to *D. magna*, being AgNPs the most toxic followed by TiO<sub>2</sub> and CeO<sub>2</sub> NPs. Interestingly, the coating of the metal oxide NPs prevented the toxic effects on this organism up to 100 mg/L. However, in the case of the coated AgNPs an increase of the toxic effects was observed, indicating differences in coating-core interaction between the metal oxide and the metal nanoparticles used in this work. Any of the TiO<sub>2</sub> and CeO<sub>2</sub> NPs was toxic to fish at the limit dose (100mg/L) nor the AgNPs up to 1.5 mg/L. The results with TiO<sub>2</sub> and CeO<sub>2</sub> NPs indicated a different taxa susceptibility to a same uncoated NP but a same sensitivity to the CIT and PEG coated NPs.

From these results, it is clear that coatings can influence the toxicity of the uncoated NPs but a general pattern of toxic behavior for these coatings within taxa and within these NPs could not be established.

**Acknowledgements** - FP7 project GUIDEnano (agreement nº 604387).



## Are there significant acute ecotoxicological effects of nanoparticles?

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The paradigm of environmental risk assessment of chemicals states that risk is to be quantified on a comparison of (predicted) environmental concentrations and (predicted) no-effect concentrations (PNECs). For the specific case of engineered nanoparticles (NPs) there is *a priori* no ground not to accept the risk assessment paradigm. In common practice, PNECs are based on chronic toxicity data generated for biota of different trophic levels. Acute effect data may be used in case of lack of chronic data, provided proper safety factors are included in the risk assessment.

Various proposals have been brought forward regarding mechanisms of interaction and subsequent toxicity of NPs. Nevertheless, actual mechanistic understanding remains limited, and there is also no simple correlation between toxic response and NP properties or NP morphology. Some information exists suggesting that subcellular endpoints, especially oxidative stress, may be more sensitive for NPs than for other more conventional contaminants. It is for instance shown that Ag NPs do not impact whole-body endpoints such as mortality and growth in the polychaete *Nereis diversicolor*, whereas subcellular endpoints like lysosomal and DNA damages were more responsive. TiO<sub>2</sub> nanoparticles have been found to cause lethal or sub-lethal effects at later stage of development only. Furthermore, effects on gene expression and on reproduction (rather than morphological and lethal effects) indicate that translocation may be the most important reason for long term toxicity. The effects on secondary organs may be more important than effects on the primary targets, whereas in the case of metallic NPs that slowly release metal ions, the effects on the primary targets were found to be leading.

These and other examples indicate a trend of subtle long-term effects of biota exposed to low levels of NPs, whereas particle specific acute toxic effects appear to be absent. On forehand, a number of factors may limit the observation of acute effects:

- A key challenge in aquatic toxicity of NP testing is that exposure often is not constant because of particle setting and other transformations that typically occur during the test, like dissolution and corona formation. Consequently, even for pristine NPs that are not altered during toxicity testing, the simple two-dimensional approach for deducing steady state mass concentrations that express the exposure dose (i.e., concentration \* exposure time = dose) is problematic for use with NPs;
- Lack of understanding of how cells sense crystals, i.e. the field of nanotoxicology needs further development;
- Lack of NP specific markers of toxicity, like phagocytosis and endocytosis-related assays to confirm the presence of particles and their subsequent adverse effects;
- Actual lack of acute effects – insufficient effective exposure and severe time restraints to reach critical levels at NP-specific sites of toxicity within biota.

In light of the factors assumed to limit the occurrence of actually occurring adverse effects of NPs, the claims of acutely induced NP effects will be discussed in this contribution. To this end, we aim to categorize the available literature with regard to a limited number of classes of NPs, assess the effective exposure, and assess whether the effects observed can indeed be classified as being particle-imposed or whether the effects are indirectly due to the addition of NPs to the exposure medium.

## TOXICITY OF COPPER OXIDE AND COPPER CARBONATE NANOPARTICLES AFTER SHORT TERM ORAL EXPOSURE

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Copper oxide nanoparticles (CuO NPs) and Copper carbonate nanoparticles (CuCO<sub>3</sub> NPs) have antimicrobial activity but only CuCO<sub>3</sub> NPs are effective as wood preservatives by impregnation. Oral ingestion by hand to mouth transfer is one of the potential routes of exposure of humans. A short term oral study (STOS) was performed to investigate whether the STOS schedule of five days treatment can be used for detection of potential toxicity of both pristine CuO NPs and CuCO<sub>3</sub> NPs. Rats were orally treated by gavage for five consecutive days and evaluated for toxicity one day after the last administration and after a recovery period of 21 days. In a preliminary experiment maximal tolerable doses were determined after a single oral administration, which was for CuO NPs 512 mg/kg and for CuCO<sub>3</sub> NPs 128 mg/kg. Using these doses as maximum dose a dose response study was performed and complete post mortem evaluation was done. For CuO only at the highest dose investigated indications for toxicity were noted by decreased white blood cell (WBC) and Red Blood Cell (RBC) parameters. Clinical chemistry showed alterations in liver enzyme levels indicating liver damage. For CuCO<sub>3</sub> after treatment the highest dose group of the recovery group showed overt toxicity and was prematurely removed from the study. In contrast to CuO for CuO<sub>3</sub> an increase in WBCs was observed indicating some inflammatory response which was absent after the recovery period in animals treated with doses of 64 mg/kg and lower. Similar to CuO clinical chemistry showed changes indicative for liver damage.

At histopathology morphologic alterations following oral administration for 5 days (once daily) with CuO<sub>3</sub> up to a dose of 128 mg/kg or 512 mg/kg CuO were observed in many organs. For CuCO<sub>3</sub> NPs pathological effects were observed starting at a dose of 64 mg/kg CuO<sub>3</sub> in the stomach and liver and at a dose of 128 mg/kg in the liver, stomach, intestines, spleen, thymus, kidneys, testes, seminal vesicles and bone marrow. Changes were mainly inflammatory (sometimes ulcerative) and/or degenerative in nature (including testes), lymphoid tissues showed lymphoid depletion and bone marrow a compensatory shift towards myeloid cells. Many of these findings did not recover after a 3 week treatment free period. Similar morphological changes were observed for CuO, in bone marrow, stomach and liver. In conclusion for CuO NPs toxic effects were noted at a dose of 512 mg/kg, while for CuCO<sub>3</sub> NPs toxic effects started at a dose of 64 mg/kg.

## Cellular and Molecular Responses to Engineered Nanomaterials On Murine Airways *In Vivo*

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Hazard assessment and toxicity studies are needed to ensure fast and reliable evaluation of potentially adverse effects of engineered nanomaterials (ENM). Airways are the most relevant route of exposure to ENM, especially in occupational settings. Studying the effects of ENM and immune response activation in the airways is necessary for creating a comprehensive understanding of the ENM-induced cellular and molecular changes. Oropharyngeal aspiration is a fast, affordable and reliable method for studying the effects of ENM *in vivo*, allowing gaining of significant quantity of data on hazards of nanomaterials (Kinaret *et al.* under revision).

We investigated the pulmonary effects of 31 carefully selected ENM (9 core materials, 8 chemistries, +/- 3 surface modifications [-COOH, -NH<sub>3</sub><sup>+</sup> and -PEG]). In order to mimic one-week occupational exposure, we treated female C57BL/6 mice 4 times for 4 consecutive days with 10 µg of ENM in 50 µl PBS by oropharyngeal aspiration. We sacrificed the mice 24 hours after the last administration and collected blood, lung tissue samples and bronchoalveolar lavage (BAL) fluid. BAL cells, including macrophages, neutrophils, eosinophils and lymphocytes, were counted with optical microscope after staining.

BAL cell counts revealed a variety of leukocyte activation depending on the material and surface functionalization. The most striking results could be detected after exposures to core and functionally modified CuO and CdTe particles, which caused high accumulation of neutrophils. Nanodiamonds, on the other hand, did not markedly activate leukocytes. Exposure to differently coated multi-walled carbon nanotubes and silver nanoparticles activated similarly neutrophils, eosinophils and lymphocytes, suggesting non-specific effects of the surface functionalization. Two different-size gold nanoparticles (5 nm and 20 nm) did not exacerbate any specific effect in any of the surface functionalizations tested. Core and PEG-functionalized spherical TiO<sub>2</sub> caused infiltration of macrophages, neutrophils and eosinophils, whereas NH<sub>3</sub>-functionalized rod-shaped TiO<sub>2</sub> induced mild accumulation of macrophages, neutrophils and lymphocytes. Interestingly, only mild elevation of macrophages was induced by COOH-coated rod-shaped TiO<sub>2</sub>.

Our results help in elucidating patterns of toxicity *in vivo*. Moreover, further characterization of these samples by transcriptome analysis will allow our data and results to be used for training the NANOSOLUTIONS ENM safety classifier.

## NEUROLOGICAL EFFECTS OF INHALED NANO-SIZED CERIUM DIOXIDE IN A MOUSE MODEL OF ALZHEIMER'S DISEASE AND WILDTYPE MICE

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Increasing evidence from toxicological and epidemiological studies indicates that the central nervous system is an important target for ambient (ultrafine) particles. Disturbance of redox-homeostasis and inflammation in the brain are proposed herein as possible mechanisms that can contribute to neurotoxic and neurodegenerative effects. Whether and how engineered nanoparticles (NPs) might play a role in the development and progression of neurodegenerative diseases such as Alzheimer's disease (AD) has not been studied so far, but forms a challenging topic of investigation and contribution to nanosafety research.

We have therefore assessed the neurological effects of subacute inhalation of cerium dioxide (CeO<sub>2</sub>) NPs with different amounts of zirconium (Zr)-doping to address the influence of redox activity, in a transgenic mouse model of AD (5xFAD) as well as in wildtype (C57BL/6J) littermates.

Mice were exposed to the NPs or clean air by nose-only inhalation over a 4-week period (4 mg/m<sup>3</sup> for 3 h/day, 5 days/week). Post-exposure, various AD-related features including altered behaviour (i.e. string suspension test, X-maze, open field test) and pathological changes in the brain such as formation of Amyloid- $\beta$  (A $\beta$ ) plaques were determined.

No major behaviour impairments were observed in association with CeO<sub>2</sub> NP exposure, neither in the AD mouse model, nor in the wild type mice. Immunohistochemical analysis revealed no accelerated formation of A $\beta$  plaques in the NP treated 5xFAD mice. The findings from present study suggest that inhaled CeO<sub>2</sub> NPs, irrespective of their redox modification, do not adversely affect the central nervous system, specifically regarding the development or progression of the neurodegenerative Alzheimer's disease.

Acknowledgment: The work leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° NMP4-LA-2013-310451.



## Splenic Capture and Degradation of Graphene Oxide *In Vivo*

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Graphene based nanomaterials are fuelling a revolution in material science and technology. Various applications in biomedicine are currently being developed using 2D materials, predominantly using graphene oxide (GO). For this reason, it is imperative to determine the biological fate of GO. We recently reported that single or few layer GO flakes of under 1  $\mu\text{m}$  in lateral dimensions are predominantly excreted in the urine, and the fraction of material that remains in the body is captured by the spleen following intravenous administration. In this study, we attempted to determine the exact localisation, long-term fate and biodegradability of GO sheets in the spleen over 9 months. Using combinations of whole body imaging using SPECT/CT, immuno-staining, cell sorting and confocal Raman mapping, we found that GO was internalised within a specific sub-population of splenic macrophages, namely the marginal zone macrophages. We used TEM coupled with electron diffraction to confirm the crystalline nature of GO flakes uptaken by splenic cells. The gradual change of the GO structure was then carefully followed by Raman spectroscopy and electron diffraction over time, providing unambiguous evidence of the biodegradation. Our findings bare important implications for the safety profile of GO materials specifically in the context of their use as nanovectors in healthcare applications.

#1520

## High-throughput analysis allows building a database to screen toxicity of nanomaterials

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The large amount of existing nanomaterials demands for rapid and reliable ways to test their potential toxicological effect on human health, preferably by means of relevant *in vitro* tests in order to reduce testing on animals.

*In vitro* tests which combine high throughput screening (HTS) with high content imaging (HCA) in an automated way allow testing numerous materials at different concentrations and different exposure times. Moreover, the automation reduces the inter-experimental variation and significantly reduces time and costs.

We present a database build using HTS/HCA summarizing, among other end points, the potential reduction of the mitochondrial membrane and membrane integrity after exposure to more than 100 NM at different concentrations on HepaRG liver cells.

The talk will discuss the data processing. The experimental set up was optimized to allow testing of stability and reliability of the data. Final potential use of the database is discussed and some observed effects are interpreted.

The authors acknowledge support from the European Commission's 7th Framework Programme project NanoMILE (Contract No. NMP4-LA-2013-310451).



## HAZARD EVALUATION IN GUIDENANO: A WEB-BASED GUIDANCE TOOL FOR RISK ASSESSMENT AND MITIGATION OF NANO-ENABLED PRODUCTS

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Within the EU FP7 project GUIDEnano, a web-based guidance tool has been developed which enables the user to evaluate and manage human and environmental health risks of nano-enabled products considering the whole product life cycle: synthesis of nanomaterials (NM), manufacturing of NM-enabled products, use, and end-of-life phase (including recycling).

The tool's quantitative human and environmental hazard evaluation is done based on information incorporated in the tool or available to the user. This information is organized in the following way:

- 1) The tool will provide previously derived hazard values (e.g. derived non effect level-DNEL, predicted non effect concentration-PNEC) for the exposure-relevant NM or similar.
- 2) Where no previously derived hazard values are available, the tool will offer the use of conservative default hazard values for general NM categories
- 3) Where conservative hazard values lead to a risk, the tool will help identify data from individual toxicity studies with the exposure relevant NM or similar.

The tool enables evaluation of each study using criteria related to:

a) similarity between the exposure-relevant NM and the tested material. The similarity assessment is based on nanomaterial properties such as coating, dissolution, primary size distribution, aggregated size distribution, shape, and level of impurities, depending on the type of study and exposure route.

b) quality of the data, based on a modified version of the ToxRTool, to be applicable to human and ecotoxicity studies performed with nanomaterials.

c) relevance of the study for each given endpoint, based on conventional use of existing test guidelines for the endpoint, and rules to derive scores in case of studies that do not follow such test guidelines.

The evaluation of these three aspects results in an overall score and only studies with NMs that have a score above a defined threshold for the relevant physicochemical properties will be considered acceptable for the hazard assessment.

Allowing the use of information from non-conventional hazard studies as well as information on NMs that are slightly different from the exposure relevant materials introduces a certain level of uncertainty. The similarity, quality and relevance scores of acceptable studies can be used to calculate associated uncertainty factors in the risk assessment.

#1498

**CYTOTOXIC AND PRO-INFLAMMATORY EFFECTS OF METAL-BASED  
NANOPARTICLES ON THP-1 MONOCYTES CHARACTERIZED BY COMBINED  
PROTEOMICS APPROACHES**

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Thorough characterization of toxic effects of nanoparticles (NP) is desirable due to the increasing risk of potential environmental contamination by NP. In the current study, we combined three recently developed proteomics approaches to assess the effects of Au, CuO, and CdTe NP on the innate immune system. The human monocyte cell line THP-1 was employed as a model of the latter. The well-known anticancer drugs camptothecin and doxorubicin were used as positive controls for cell death and lipopolysaccharide (LPS) was chosen as a positive control for pro-inflammatory activation. The anticancer drugs and the three NP were used at concentrations inducing 50±10% cell death in THP-1 cells. The proteome changes were measured by LC-MS/MS and assessed via uni-, multi-variate and pathway analyses. Despite equivalent overall toxicity effect, the three NP induced distinctly different proteomics signatures, with the strongest effect being induced by CdTe NP, followed by CuO NP. The CdTe toxicity mechanism involves down-regulation of topoisomerases, and is likely due to interference with DNA of chromatin proteins. The effect of CuO NP is most reminiscent of oxidative stress, and involves up-regulation of proteins involved in heat response. The gold NP showed the weakest effect and induced up-regulation of the inflammatory mediator, NF-κB and a number of proteins related to energy metabolism, consistent with pro-inflammatory activation. Thermal proteome profiling suggested that NF-κB up-regulation could be the result of down-regulation of its inhibitor TIPE2 induced by direct binding to gold NP. Overall, the combined proteomics approach described here can be used to characterize the effects of NP on immune cells.

## IN SILICO CAPTURE OF TOXICITY EFFECTS RELATED TO NANOMATERIALS USING A NOVEL PREDICTIVE TOXICOGENOMICS SPACE TOOL

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Toxicogenomics is an expanding research discipline with potential to aid in safety evaluation of engineered nanomaterials (ENM). We generated recently a comprehensive "predictive toxicogenomics space" (PTGS) description from performing a "big data compacting and data fusion"- analysis of transcriptomics and cellular toxicity data generated with >1000 FDA-approved drugs. Based on the capture of possibly a majority of existing cellular toxicity reactions, we hypothesized that the PTGS should be applicable to analyze ENMs toxicity. On this basis, we initially mined the overlap of toxicity mechanisms in the PTGS relative an existing summary of biological processes studied in nanotoxicology literature existing in PubMed (summarized by Klaper et al, Analyst, 2014). Three types of gene set overlap analysis, including PTGS relative the Gene Ontology, the Ingenuity ToxLists or the MSigDB 5.1 Hallmarks, pointed collectively to coverage of 20 of 21 nanotoxicity-associated processes by the PTGS. Seven of the 21 processes were covered in each of the analysis, including extensively transductional signaling, cell signaling and receptor-mediated response. Being the most studied toxicity mechanisms, oxidative stress, apoptosis and cell proliferation were also covered in each of the analysis. Analysis relative specific subcomponents in the PTGS pointed further to connection of nanomaterials to these toxicity mechanisms. Choosing next a specific case study with transcriptomics data, we assessed for the ability of the PTGS to capture the relative influence of surface modification of polyamidoamine (PAMAM) dendrimers (hydroxyl- vs. amine-functionalization) (Feliu/Kohonen et al, ACS Nano, 2015). Equimolar concentrations of non-cytotoxic levels (deemed so from absence of membrane damage in the original study) of the hydroxyl and amine-variants altered 7% and 32% of the PTGS genes, respectively, pointing to a relatively higher growth inhibitory activity of the amine-functionalized variant. Analysis of PTGS subcomponents further indicated induction of stress reactions and cell cycle arrest of the latter variant. We conclude based on these initial studies that PTGS scoring potentially serves to capture a majority of potential toxicity effects exerted by ENMs. The selected case study additionally indicated that PTGS scoring enables high sensitivity for detection of toxicity-associated influences.

## NANOPARTICLE-INDUCED PROTEIN FIBRILLATION AND NEURODEGENERATION CASCADES IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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By unbiased proteomics we identify the gene ontology group of 'protein folding, proteolysis and stress response' as a major target of silica nanoparticles (NPs) in adult hermaphrodite nematodes. Segregation of decisive proteins such as heat shock proteins and components of the 19S proteasome regulator into a silica NP-induced aggregome network promotes widespread amyloid-like protein fibrillation that manifests in nucleoli of the worm's intestinal cells, body wall muscle cells and axons of single serotonergic hermaphrodite specific neurons (HSNs). The pathway of protein homeostasis was validated as a major target of silica nanoparticles by behavioral phenotyping, since inhibitors of amyloid formation rescued nanoparticle-induced defects of locomotory patterns and egg laying. In HSN neurons presynaptic accumulation of serotonin, e.g. disturbed axonal transport reduces the capacity for neurotransmission and impairs the downstream phenotype of egg laying. The results suggest that in *C. elegans* silica nanoparticles promote a cascade of events including disturbance of protein homeostasis, widespread amyloid protein aggregation and inhibition of neural function that controls worm behavior. This neuromuscular cascade is practical for medium throughput screening of nanomaterial safety. Environmental exposure scenarios, the habitats of wild *C. elegans* and their coping capacities with impaired neural functions will be discussed.

## A PATHWAY TOOL FOR APPLYING “BIG DATA” IN NANOSAFETY-FOCUSED READ ACROSS AND ADVERSE OUTCOME STUDIES

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A focus of several ongoing European Nano Safety Cluster projects is to compress and interpret omics-generated results into useful predictive biological pathway-based analysis concepts and tools. We hypothesized that focused subsets of relevant curated adverse outcome-specific pathways may allow for deepened risk analysis. Using the open collaborative pathway platform WikiPathways, we created an ENM portal enabling community-based annotation of toxicity pathways and adverse outcomes relevant to nanosafety (<http://www.wikipathways.org/index.php/Portal:Nanomaterials>). Pulmonary fibrosis, a serious chronic respiratory condition induced by a wide variety of environmental and occupational exposures such as asbestos, silica dust and potentially also multi-walled carbon nanotubes, was selected to test the concept. Information and data related to lung fibrosis and ENMs were extracted and mined from public data sources. Using the bioinformatics tool PathVisio, the following steps generated a first description of a bioinformatically employable WikiPathway for this adverse outcome: a general scheme for the molecular processes of lung fibrosis was drawn based on selected review articles; sixty-four lung fibrosis associated genes with direct association were obtained from the Comparative Toxicogenomics Database; interactions between the 64 genes were obtained from the GeneMANIA database; highly interactive groups of genes were analysed for pathway or gene ontology enrichment using the Consensus Pathway Database; and finally, the elements of the pathway were linked to key events in two recently published putative adverse outcome pathways (AOPs) for pulmonary fibrosis. Transcriptomics data from pulmonary fibrosis patients and human cells exposed *in vitro* to various agents, including diverse ENM, but also fibrosis-associated chemicals and particles, was analysed and visualized using the novel WikiPathway. Overall, this workflow applied and compressed data from three lung fibrosis pathway-focused publications relative the three selected databases. We conclude based on these initial analyses that this novel workflow concept serves diversely for: i) gene set enrichment analysis, ii) pathway enrichment analysis among differentially expressed genes, iii) integration into AOP-based testing strategies, iv) application as descriptors in (Q)SAR approaches, and finally, iv) for grouping and read across among ENMs, coupled to identification of specific pathway-activating ENMs. Our further work aims to demonstrate the translational use of the novel tool generating concept for ENM safety assessment.

## Omics workflows implemented in the NANOSOLUTIONS Project

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Theme: Systems biology approaches in nanosafety

### Abstract

The main aim of the NANOSOLUTIONS project is to generate a classifier, based on a set of biomarkers that can predict the toxicity of newly developed engineered nanomaterials (ENM). To identify potential biomarkers, the NANOSOLUTIONS consortium has generated a set of 31 materials with different composition (Ag, Au, Cu, TiO<sub>2</sub>, carbon, and CdTe), shape (spheres vs rods), size (5 vs 20 nm) and surface chemistry (core, COOH, NH<sub>2</sub> and PEG). Bacteria, human cell lines, and mice were treated with these ENMs in triplicates, and investigated using various Omics techniques to detect ENM-induced changes in transcriptome and proteome. The *in vitro* cell models, ENM doses and exposure times, were chosen based on discussions within consortium. Here we describe the different Omics pipelines and platforms used to determine the transcriptomes (RNA and miRNA sequencing) and proteomes of the exposed materials. The same pools of total RNA were used for both RNA sequencing and miRNA analysis, while independent cells were prepared for the proteomics analysis.

Two human cell lines were treated with a dose of EC10 of all 31 ENMs for 24 hours. All 31 ENMs were tested also on asthma using a mouse model. Sequencing libraries were prepared according to modified protocol for single-cell tagged reverse transcription (STRT), where 10ng RNA/sample was used. All samples were sequenced on three lanes with Illumina's HiSeq 2000 sequencing system. On average, we received 2.5M reads per sample for THP.1 and 1.9M for BEAS-2B, which were at the 5'-UTR or just upstream of protein coding genes, i.e. intact mRNAs. The number of reads for each sample was in normal range. The validation of the sequencing results from THP.1 and BEAS-2B samples was done with Agilent's SurePrint G3 Human Gene Expression v3 8x60K Microarrays.

To study genome-wide miRNA expression, TruSeq small RNA next generation sequencing (NGS) was used. In addition to profiling of known miRNAs, this system enables discovery and profiling of novel miRNAs and other non-coding RNA types. The miRNA sequencing was done on Illumina's HiSeq 2500 and HiSeq 3000 NGS platforms. In total, 214 microRNA samples were sequenced (3.74 million reads/sample). After trimming the 3' adaptor, the reads were processed by the miRDeep2 package to identify and quantify known and

novel microRNA. In order to have general mapping statistics of the microRNA-seq data, the reads were also aligned to the human genome (hg19) using Tophat2. 45-60% of the trimmed reads aligned to the whole genome. Of the aligned reads, 42% mapped to 1612 of 2588 (50%) annotated miRNAs of human miRBase. The microRNA expression output by miRDeep2 was normalized using the Trimmed Mean of M-values, or TMM method, which is implemented in the edgeR Bioconductor package.

For proteomics, one negative (media) and two positive (LPS and STS) controls were included in the analysis. All samples were processed using standardized protocols. After lysis and protein extraction, all samples were reduced, alkylated and digested by trypsin using a MassPREP robotic protein-handling system. The peptides were analyzed by nanoLC-MS/MS using a NanoUltimate 3000 coupled on-line to a Q Exactive Plus mass spectrometer. The peptides were separated using a C18 reverse column using a 2 hour gradient. The proteins were quantified using MaxQuant. In total, 315 samples (102 THP.1, 144 BEAS-2B and 69 *E.coli*) were analyzed, identifying and quantifying 2,720 (THP.1), 4,374 (BEAS-2B) and 1,632 (*E. coli*) proteins, with at least 2 peptides and at 1% FDR.



## MODELLING BIONANO INTERACTIONS FOR PREDICTIVE TOXICOLOGY

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Over the last decade, in vitro and in vivo experiments have produced significant amount of veritable information that can be integrated into theoretical models with the aim of predicting possible health and environmental effects of engineered nanoparticles (NP). However, even the most systematic studies often leave the question of precise toxicity mechanisms associated with NPs wide open. One can speculate that the toxic effects can emerge either from membrane damage or from interaction of NPs, once they are inside the cell/tissue, with the internal cell machinery and signaling pathways. Therefore, NP ability to penetrate the cells, bind to key biomolecules, or perturb the normal pathways, either by physical (e.g. membrane disruption) or biochemical mechanisms (e.g. production of reactive oxygen species) can be important. Significant research efforts were invested recently into nanotoxicology in an attempt to identify nanomaterials' properties of concern, which can be responsible for these interactions, and model their relationships with adverse outcomes.

While statistical analysis allows one to relate the physical and chemical descriptors of the materials to certain toxicity endpoints, the mechanisms of action are not always known. The quantitative relations between the descriptors and the effect can only be deduced once we have a clear picture of all stages of interaction between the foreign agent and the biological tissue and of all stages of the systemic transport at the mechanistic level. The identification of molecular or nanomaterial properties responsible for the uptake or hazard can be facilitated by establishing the molecular initiating events or key events in each adverse outcome pathway and by mechanistic modelling of the underlying processes. A development of an intelligent, mechanism-aware testing strategy may require an *identification of novel mechanistic endpoints*. On the other hand, to build a successful predictive model, one should develop a new language suitable for description of bionano interactions and identify the *relevant NP and biomolecule descriptors*. In most cases, the standard physicochemical descriptors of NPs are not fit for this purpose as they do not allow to predict whether a specific NP would bind to the membrane, adsorb or deform the key molecule.

To create a basis for grouping the nanomaterials, read-across, and for development of quantitative models of toxicity, we propose to form a database of bionano interactions in addition to common physicochemical descriptors. We develop a computational scheme for a fast evaluation NP-biomolecule interactions. We use a bottom-up molecular simulation approach, which relates the advanced protein and lipid descriptors (sequence and structure descriptors) with basic molecular interactions at the interface. We address protein adsorption on metals, oxides, and carbon-based materials. We present: (1) a principal scheme of the model required for understanding of protein adsorption at various solid interfaces; (2) propose a set of advanced NP descriptors (e.g. hydration energy, ionisation potential, conduction band gap, Hamaker constant, etc.) and show how they can be calculated, (3) the scheme of model parameterisation using experiment or simulation, and (4) a bio/nanoinformatics-based scheme to predict free energy of adsorption of globular proteins at liquid/solid interfaces and (5) a way of ranking adsorption propensity of proteins. We also construct and validate a method of modelling the adsorption kinetics and competitive adsorption of proteins on NPs and formation of NP protein corona. We quantify the influence of NP surface curvature, charge and coating on the adsorption energies for important plasma proteins and rank the proteins by their binding

affinity to the NPs. We show what properties of NPs can be most promising for predictive modelling of bionano interface and toxicity mechanisms.

This work is supported by EU H2020 project SmartNanoTox under grant agreement No. 686098.

**#1642**

**Answering Scientific Questions with linked European nanosafety data**

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**ENM SAFETY CLASSIFIER – A multi-view feature selection and classification algorithm for prediction of engineered nanomaterials (ENM) safety.**

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We present the computational infrastructure of the NANOSOLUTIONS ENM safety classifier. It is built upon computational ensemble classification systems capable to predict the safety of ENM based on selected minimal but most informative set of intrinsic and mode of action features. We trained the classifier on a comprehensive set of data layers, including physico-chemical characteristics, transcriptomics, proteomics, immunotoxicity and genotoxicity retrieved from 31 carefully selected ENM tested in a multitude of *in vitro* and *in vivo* set ups. The main components of the proposed computational infrastructure are class discovery and ENM classification. Class discovery is accomplished by multi-view clustering and is used to suggest sensitive ENM grouping. ENM classification is implemented as an ensemble of machine-learning techniques. The proposed multi-view approach uses a three-level hierarchical ensemble clustering method that i) compiles similarity matrix for each data source separately, ii) combines the similarity matrices into a unified model comprising information from clustering results of different data types, and iii) agglomerates the unified models into enhanced consensus clustering across different data layers and exposure set ups. The final ENM categorization is used to guide the classification tasks. The ENM safety classifier allows to independently search for optimal intermediate models within each data layer, which are then systematically combined into optimal hybrid predictive models. This strategy offers several advantages: i) it preserves data-specific properties, ii) it exploits different available data types to improve classification accuracy, iii) it handles missing views, and iv) it attenuates biases given by unbalanced data. Our computational infrastructure is able to evolve by learning from next generations of data and achieve better classification of ENM safety in the future.

[Supported by FP7-309329 NANOSOLUTIONS]

**#1513**

## **Event Correlations in the Apoptosis Signaling Cascade gained from Single Cell Time-lapsed Analysis**

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Cell fate decisions like apoptosis are outcomes of intricate signaling cascades being highly heterogeneous both in strength and dynamics when observed at the single-cell level.<sup>1,2</sup> Programmed cell death is induced by various pathways each showing organelle-specific signals as the central integrator of apoptosis. Lysosomes are discussed as triggers for cell death but also mitochondria or caspases are believed to be central executioners of apoptosis. The symptomatic order and timing of events in the signaling cascades during programmed cell death, however, are not systematically captured. Here, we monitor the time lines of apoptotic signals in automated time-lapse microscopy in combination with single cell micro-arrays. By pair wise labeling we establish sequences of events with high temporal resolution using time correlation analysis. In particular lysosomal breakage (LMP), mitochondrial outer membrane permeabilization (MOMP), increase of ROS level (ROS), caspase 3 activation (CASP), exposure of phosphatidylserine to the exterior of the membrane (PhS-FLIP) and loss of plasma membrane integrity accompanied with nucleus staining (PMP) are studied. For cell death induced by amino-functionalized polystyrene nanoparticles, staurosporine and the FAS-ligand we find distinct timing in the signal pathway and distinct variance in event correlations indicative of cross-talk in the apoptotic pathways. Hence multi-dimensional time-correlation provides a dynamic fingerprint of signaling pathways that is likely to be useful in nanosafety to identify the effect of nanoparticles.

1 Röttgermann P., Dawson K. and Rädler J., Time-Resolved Study of Nanoparticle Induced Apoptosis Using Microfabricated Single Cell Arrays, Microarrays, 2016

2 Röttgermann P., Piera Alberola A., Rädler J., Cellular Self-Organization on Microstructured Surfaces, Soft Matter, 2014.

## A NETWORK BASED DATA INTEGRATION TOOL FOR THE CONTEXTUALIZATION OF ENGINEERED NANOMATERIALS (ENM) MODE OF ACTION

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In recent years, omics technologies have been increasingly used to thoroughly characterize the engineered nanomaterials (ENM) molecular mode of action. It is possible to contextualize the molecular effects of different types of perturbations by comparing their patterns of alterations. While this approach has been successfully used for drug repositioning, it is still missing to date a comprehensive contextualization of the ENM mode of action.

We developed INSIdE nano (Integrated Network of Systems biology Effects of nanomaterials), a novel computational framework for omics data integration able to highlight significant similarities between ENM, drugs, chemicals, and diseases, depending on their effects on the transcriptome. Based on the expression signature, associated to each phenotype, the strength of similarity between each pair of perturbations was evaluated and used to build a large network of phenotypes. In order to ensure the usability of INSIdE nano, we developed a robust and scalable computational infrastructure to scan this large phenotypic network and we built a web-based effective graphic user interface.

Our evaluation of INSIdE nano confirmed that it highlights known disease-drug and disease- chemical connections. Moreover, disease similarities are in agreement with the information based on their clinical features, as well as drugs and chemicals, mirroring their resemblance based on the chemical structure. Altogether, our results suggest that INSIdE nano can also be successfully used to contextualize the molecular effects of ENM and infer their connections to other better studied phenotypes, speeding up their safety assessment as well as opening new perspectives concerning their usefulness in biomedicine.

**MAPPING THE NANOSAFETY CLUSTER DATA**

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Nanomaterial safety assessment has become an important task following the production growth of engineered nanomaterials (ENMs) and the increased interest for ENMs from various academic, industry and regulatory parties. A number of challenges exist in nanomaterials data representation and integration mainly due to the data complexity and origination of ENM information from diverse sources. We have recently described eNanoMapper database [1] as part of the computational infrastructure for toxicological data management of engineered materials, developed within eNanoMapper project [2].

The eNanoMapper prototype database is publicly available at <http://data.enanomapper.net>, demonstrating the integration of data from multiple sources, using the common data model and Application Programming Interface. The supported import formats are IUCLID5 files (OECD HT), semantic format (RDF) and custom spreadsheet templates. The latter accommodates the preferred approach for data gathering for the majority of the NanoSafety Cluster projects and is enabled by a configurable parser mapping the the custom spreadsheet organization into the internal eNanoMapper storage components through external configuration file. Import of spreadsheet data and other data formats, generated by a number of NanoSafety Cluster projects is currently ongoing. The export formats have been extended with the new ISA JSON format, following the most recent ISA specification.

Defining templates for data gathering is a common activity for most of the NanoSafety Cluster projects usually resulting in modified Excel spreadsheets. In order to help avoiding the incompatibility issues, we present a tool for template generation, based on templates released under open license by JRC under the framework of the NANoREG project [3]. A number of physchem, *in-vitro* and *in-vivo* assays are supported and using feedback from users we added and extended existing information about different aspects of nanosafety, e.g. environmental exposure, cell culture assays, cellular and animal models, nanomaterial production features, and nanomaterial ageing.

Finally, the data can be accessed programmatically via the application programming interface as well as via user friendly search interface at <https://search.data.enanomapper.net>. The search application is powered by a free text search engine and eNanoMapper ontology and was improved over the last year based on user feedback. The search function allows now multiple filtering for information. It is possible to stack filters for e.g. nanomaterial type, cell model and assay. For certain features of nanoparticles it is possible to filter for a certain range of results, e.g. nanoparticles of a size between 5 and 10 nm.

eNanoMapper is supported by European Commission 7th Framework Programme for Research and Technological Development Grant (Grant agreement no: 604134).

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#1578

## RAPID HIGH THROUGHPUT SCREENING PLATFORM FOR NANOMATERIALS

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A high throughput nanomaterial screening system is being developed within the framework of the H2020 project HISENTS. The initial system being built is an extension of the very successful FP7 ENNSATOX technology. The device is composed of a flow cell with individual biomembrane-like sensor element interfaced to miniature reservoirs by microfluidic flow networks. The endpoint is registered as damage to the continually replaceable sensor element and recorded electronically.

Throughout the duration of the project the single modular system will be further miniaturised and extended to multimodular. Additional sensor elements will be incorporated representing a variety of critical physiological functions.

This talk will initially describe the structure, workings and performance of the platform recorded against standard intercalibrant water soluble toxins, where limits of detection (LoD) and response “fingerprints” will be shown for each toxin. The response will be compared to very standard toxicity predictors for individual compounds such as log *P* and, to toxicity to fish. The mechanistic details of the precise molecular event which the sensor element actually measures will be discussed for each compound-membrane interaction. The second part of the talk will describe the application of the screening platform to gold (Au) and silver (Ag) nanomaterials (NM). The synthesis and characterisation of the NM is detailed and the screening results will be reported. In particular the relation between the extent of the electronic response and the **size** and **shape** of the NM particles will be shown and related to the mechanism of interaction of the particle with the biomembrane-like sensor element. It is interesting that while it is intuitive that smaller particles have a stronger interaction than larger particles with the membrane surface, the interaction is also dependent on particle shape. Results will also define how the surface of the particle which is determined by its synthetic route relates to its biomembrane-interaction.

In the final few slides, future plans will be shown for miniaturising the platform and extending it from single modular to multimodular including more specific sensing modules.



#1627

## SMARTNANOTOX

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This presentation will describe the H2020 project entitled « Smart Tools for Gauging Nano Hazards ».

A definitive conclusion about the dangers associated with human or animal exposure to a particular nanomaterial can currently be made upon complex and costly procedures including complete NM characterisation with consequent careful and well-controlled *in vivo* experiments. A significant progress in the ability of the robust nanotoxicity prediction can be achieved using modern approaches based on one hand on systems biology, on another hand on statistical and other computational methods of analysis. In this project, using a comprehensive self-consistent study, which includes *in-vivo*, *in vitro* and *in silicio* research, we address main respiratory toxicity pathways for representative set of nanomaterials, identify the mechanistic key events of the pathways, and relate them to interactions at bionano interface *via* careful post-uptake nanoparticle characterisation and molecular modelling. This approach will allow us to formulate novel set of toxicological mechanism-aware end-points that can be assessed in by means of economic and straightforward tests. Using the exhaustive list of end-points and pathways for the selected nanomaterials and exposure routs, we will enable clear discrimination between different pathways and relate the toxicity pathway to the properties of the material *via* intelligent QSARs. If successful, this approach will allow grouping of materials based on their ability to produce the pathway-relevant key events, identification of properties of concern for new materials, and will help to reduce the need for blanket toxicity testing and animal testing in the future.

Finally, preliminary results of WP1 on the *in vitro* metal oxides nanoparticles exposure of macrophages will be described.

**#1652**

**-LAZAR: A FRAMEWORK FOR NANOPARTICLE READ ACROSS RISK  
ASSESSMENT**

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lazar is a modular framework for read across predictions of chemical toxicities. Within the eNanoMapper project lazar was extended with capabilities to handle nanomaterial data, interfaces to other eNanoMapper services (databases and ontologies) and a stable and user-friendly graphical interface for nanoparticle read-across predictions.

In this presentation we will give (I) an overview of the lazar framework and its integration into the eNanoMapper infrastructure, (ii) present validation results with different algorithms and nanoparticle descriptors, and (iii) give an online demonstration of the nano-lazar webinterface.

**#1636**

**A METHODOLOGICAL APPROACH FOR THE SAFE DESIGN AND PUTTING INTO SERVICE OF NEW PRODUCTION PROCESSES FOR MANUFACTURING CNT-NANO-ENABLED PRODUCTS**

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The production of new ENMs and nano-enabled products (NEPs), involves in parallel the design and commissioning of new sustainable manufacturing processes or the modification of existing ones to adapt them to new manufacturing needs. Scaling from laboratory plants to commercial production processes represents a drastic leap, both in terms of technology and legal requirements.

The European legislation governing the free movement of new machinery within the European market is the [Machinery Directive 2006/42/EC](#) (MD). The Directive sets out the mandatory Essential Health and Safety Requirements (EHSRs) for machinery while detailed technical specifications for fulfilling these requirements are given in European harmonized standards.

Prevention through Design (PtD), Safe-by-Design (SbD) or Safety Integration (SI) are similar concepts that refer to design out hazards or minimize risks early in the design process. Principles of Safety Integration - sometimes referred to as SbD - are an essential section of the MD and guide the safe design and construction of new machinery. Risk assessment is a basic requirement of the MD and also the key tool for the safe design of machinery. However harmonized standards are not available to facilitate the process of risk assessment of new ENMs & NEPs manufacturing machinery.

Project PLATFORM is a H2020 Research and Innovation action (GA 646307) aimed to operate, in the short-term, three new Pilot Plants (PPPs) for the industrial production and commercialization of NEPs (buckypapers, treated prepregs, doped veils), for the aeronautics and automotive European industries. PPPs are not required to comply with the provisions of the MD until they are put into service in 2020 (beyond project completion), but at that time, all applicable requirements of the MD will be mandatory to PPPs.

This paper shows the methodological approach followed by PLATFORM for the safe design and putting into service of PPPs according to the EHSRs of the MD, in order to prevent and reduce the risks to health from hazardous substances emitted by PPPs (airborne emissions, wastewaters, wastes), facilitate the CE marking in 2020 and avoid potential economic costs associated with future re-adaptations or modifications needed to ensure compliance with the MD when PPPs are putting into service.

The main challenge has been the integration of all nanosafety issues in the well-established risk assessment process established by the MD. The risk assessment of PPPs has been facilitated by PLATFORM – SbD tool, a simple and friendly Microsoft Excel tool developed by the project. Since the commissioning of PPPs involves design aspects that go beyond nanosafety issues, this tool can be applied to the overall risk assessment of PPPs or only for risks related to the use and handling of ENMs and NEPs.

## SIZE DEPENDENT INTERNALISATION PROFILE OF PEGYLATED MULTIWALLED CARBON NANOTUBES AND THE IMPACT OF PROTEIN ADSORPTION

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Carbon nanotubes exhibit unique physical and chemical properties that make them promising candidates for biomedical applications. A key parameter for their medical translation is surface functionalization in order to overcome the low dispersibility in biological fluids. PEGylation of multiwalled carbon nanotubes (MWCNT) via noncovalent functionalization with PEGylated phospholipids surprisingly increased their dispersibility and stability. However, the effect this functionalization has on the interaction of MWCNTs with plasma proteins and subsequent internalisation and cytotoxicity in vitro and biodistribution in vivo is not very well understood. For this purpose, we synthesized two size ranges of MWCNT (long MWCNT, 0.5-2.5µm and short MWCNT <0.5 µm) and functionalized them with DSPE-PEG2000 (MWCNT-PEG) in order to investigate the effect of MWCNT length on their internalisation and cytotoxicity in the presence or absence of plasma proteins in vitro (MCF-7 cell line) and in vivo (CD-1 mice). TEM imaging confirmed that PEGylation of both short and long MWCNT significantly improved their dispersibility in aqueous solutions. PEGylation of MWCNT resulted in significant increase in surface abundance of C-C and C-OH as indicated by XPS data and revealed the presence of ~30% of surface oxygen compared to <1% for non-PEGylated MWCNT. Raman spectroscopy confirmed that both types of MWCNT maintained their Raman signature after PEGylation.

We studied the uptake and cytotoxicity of the short and long MWCNT-PEG in vitro as well as the biodistribution and clearance of the material from the blood in vivo. Using conventional and imaging flow cytometry we were able to accurately determine the uptake of MWCNTs by MCF-7 cells. We applied TEM in order to confirm the results obtained by flow cytometry. We observed a remarkable difference in the uptake profiles of the studied MWCNTs. Long MWCNT-PEG exhibited extremely low uptake by MCF-7 cells compared to the short MWCNT-PEG that displayed a dose dependent uptake by the cells. Propidium iodide staining of dead cells revealed that MWCNT-PEG did not impair cellular viability. Effect of the serum proteins on the uptake of MWCNT-PEG was also studied. We showed that the presence of proteins decreased cellular uptake of short MWCNT-PEG, but did not have any effect on the uptake of long MWCNT-PEG. In

agreement with the in vitro data, we observed rapid cellular uptake of short MWCNT-PEG by the cells from reticuloendothelial system after in vivo administration and subsequently reflected in their ultrafast removal from the blood circulation. These results were confirmed by direct probing of Raman signatures for the MWCNTs in mice tissues (lung, liver and spleen). In conclusion, this study demonstrated that the size of PEGylated MWCNT is a determinant factor for their behaviour both in vitro and in vivo and should be taken into consideration in the design of MWCNT for biomedical applications.

# INFLUENCES OF GOLD NANOPARTICLES WITH SURFACE-ANCHORED CHIRAL POLY(ACRYLOYL-L(D)-VALINE) ON PROTEIN ADSORPTION, CELLULAR UPTAKE AND CYTOTOXICITY

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The design of smart multifunctional nanoparticles (NPs) for targeted therapies and intracellular imaging requires insight understanding of cellular uptake of NPs and their intracellular fates. Upon NPs encounter biological fluids, their surface shall be inevitably adsorbed with proteins to form a protein corona. Although many studies have demonstrated that surface modification of AuNPs with biomolecules endows them with various biological functionalities, the impact on living organisms of the chirality of the surface molecules at nanoscale has not been disclosed. Herein the effects of AuNPs capped with different chiral forms of poly(acryloyl-L(D)-valine) (L(D)-PAV) on protein adsorption and cellular uptake are investigated. To synthesize the PAV molecules, the monomers of L(D)-acryloylated amino acids were synthesized and polymerized via the reversible addition-fragmentation chain transfer (RAFT) polymerization method. Detail characterizations demonstrated that the L-PAV-AuNPs and D-PAV-AuNPs have identical physicochemical properties except of the reverse molecular chirality. The PAV-AuNPs were mainly distributed in cytoplasm, regardless of chirality. With the concentration of FBS increasing, the adsorbed amount of proteins on the PAV-AuNPs increased, and the internalized amount of PAV-AuNPs by A549 cells was decreased. In DMEM and 10% FBS/DMEM, A549 cells took up higher amount of D-PAV-AuNPs. However, in 50% FBS/DMEM and 20 mg/mL BSA/DMEM, the internalized amount of L-PAV-AuNPs and D-PAV-AuNPs was chirality-independent. The cytotoxicity is positively correlated with the cellular uptake amount, and thereby the production of intracellular reactive oxygen species (ROS). This finding discloses the importance of molecular chirality on the protein adsorption and thereby the cellular uptake of nanomaterials.

The molecular chirality on AuNPs acts as a direct regulator of cellular uptake, but the effect of chirality on cellular uptake disappears when the NPs are covered by proteins, especially BSA. Identification of this chirality-dependent cellular uptake of NPs provides a new idea that chiral effect can act as a novel strategy for designing bio-interface materials and may open a new avenue for further development of gold nanoparticles for biomedical applications.

**Acknowledgment:** This study is financially supported by the Natural Science Foundation of China (51120135001, 21374097), and the Key Science Technology Innovation Team of Zhejiang Province (2013TD02).

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## IMPORTANCE OF MULTI-METHOD CHARACTERISATION APPROACH IN THE DEVELOPMENT AND BEHAVIOURAL UNDERSTANDING OF NANOMATERIAL LIBRARIES FOR NANOSAFETY STUDIES : POLYVINYLPIRROLIDONE (PVP) CAPPED METAL OXIDE NANOPARTICLES

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The potential long-term environmental impact of manufactured nanomaterials (MNMs) remains poorly understood, and the need to better predict MNM fate and transformations and chronic effects is particularly urgent. Despite this, the field has yet to advance sufficiently due to challenges in characterisation and in linking systematically physicochemical properties to toxicity. It is therefore essential to develop reference nanomaterial (NM) libraries, with a view to develop a body of characterisation and (eco)toxicity data, to enable a mechanistic understanding of toxicity and ultimately to facilitate a move towards computational prediction of NM behaviour and fate.

The aim of this work was to develop and fully characterise a library of comparable nanoparticles with a range of core chemistries, but the same capping agent and size range, for use in future studies to test the hypothesis that the core chemistry is a primary factor in controlling toxicity. A key consideration for the synthesis method is that it is easy to use, reproducible and yielding high quantities of good quality particles. A simple single stage reflux method, involving an aqueous metal nitrate precursor, was tested with the aim to develop a range of monodisperse metal oxides NMs with controllable sizes. The novelty of this synthesis approach is the requirement for the method to work with a range of metal salts, something that is not normally needed for commercial applications of NMs. The library based on the PVP capped ceria synthesis protocol of Merrifield et al. (2013) was adapted and finally contained the following NMs: 10 K, 40 K and 360 K PVP capped ceria, zinc oxide and copper oxide (9 NMs in total). Physico-chemical characterisation methods including DLS (both size and zeta measurements), UV/Vis, XPS, ICP-OES, FT-IR, TEM, STEM, EDX, XAS and EELS, revealed the success of the synthesis by confirming the presence of the metal oxide core and showed that the synthesis is easily adaptable and scalable. More importantly, multi-method characterisation avoided misinterpretation of valency information as results from various techniques revealed limitations in what can be gleaned from each technique. Results showed that a common mechanism hypothesis holds true. PVP was found to play a significant role in the synthesis of the NMs. Additionally PVP influenced both the physical and chemical properties of the NMs. Furthermore, toxicity results produced through novel high throughput screening, show toxicity for some metal oxide variants but no toxicity for others samples. The next stage of the research involves obtaining the toxicity data of the entire library and correlating it with metal core speciation. Finally, the NM library has the potential to be extended further to other NMs such as other rare earth oxides.

**Acknowledgement:** The authors would like to thank QNano, NanoMILE and Endeavour Scholarships Scheme (Group B) for partly financing the research as well as Diamond Light Source for access to beamline I18 (proposal no. SP12760).

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## TRANSLOCATION AND QUANTIFICATION OF NANOMATERIALS AT SINGLE CELL LEVEL BY MEANS OF LABEL-FREE IMAGING AND DOSIMETRIC TECHNIQUES

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Increasing applications of engineered nanomaterials (ENMs) in the industry and private consumption demand the thorough identification of hazards and potential adverse effects on human and environment. These adverse effects include the ability of ENM to induce damage at the cellular, tissue, or organism levels by interacting with cellular structures. Modifying the surface chemistry of nanomaterials is of prime importance to control and understand the potentially toxic impact of nanomaterials, their translocation and localization.

The translocation of ENMs across plasma membranes was studied in human lung adenocarcinoma epithelial cells (A549) by means of label-free imaging methods based on elemental and molecule analysis. Micro-Proton induced X-ray emission ( $\mu$ PIXE) and micro-Rutherford backscattering ( $\mu$ RBS) were used simultaneously at the Leipzig Ion Nanoprobe LIPSION for cell analysis. These two IBM techniques provide unique and powerful tools for element dosimetry and spatially resolved elemental analysis. Cellular response, quantification of ENM uptake and their distribution at the single cell level were performed for  $\text{CeO}_2$  as well as for CuO Core, CuO  $\text{NH}_3^+$ , CuO PEG and CuO COOH. It was shown that the uptake of the CuO ENMs by cells is influenced by their surface chemical properties. Additionally the confocal Raman microscopy (CRM) was applied for 3D visualization of ENMs at subcellular level. The co-localization of ENMs with cell compartments as well as with biomolecules was analysed.

Correlation of the intracellular uptake with the toxicological response was studied. The uptake and toxicity studies revealed that PEGylation as well as carboxylation of CuO has a protective potential even at higher uptake rates.

The results of ENM uptake *in vitro* were compared and correlated with *in vivo* study. For this purpose  $\text{CeO}_2$  uptake, localization and distribution in lung tissues of Wistar rats exposed to  $25 \text{ mg/m}^3$   $\text{CeO}_2$  over 28 days were analysed.

## AN IN VITRO AND IN VIVO STRATEGY TO PRIORITISE NANOMATERIALS FOR TOXICITY TESTING AND TO INFORM SAFETY-BY-DESIGN

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In order to assess the current state-of-the-art, and to prevent repetition of existing data, a literature search was employed to ascertain the hazard information available for the priority nanomaterials (NMs) to be assessed in SUN. However, since there is a significant delay in publishing data following generation, an audit of relevant data from other sources such as reports and deliverables from other projects was conducted. Based upon this information TiO<sub>2</sub> and Ag NMs were not tested further in this project, instead existing data was used for risk assessment. In contrast CuO was identified as lacking hazard information. Two *in vitro* models were employed, macrophages to represent immune cells, and hepatocytes to represent a non-immune target cell from an organ known to accumulate particles. An *in vitro* assessment of both the particles cytotoxicity and pro-inflammatory response was conducted using protocols from previous EU projects, thereby allowing comparison of data across projects. The data generated indicated that CuO NMs were relatively toxic, and this data was used to refine subsequent animal experiments in terms of the doses to be tested. A short term inhalation study demonstrated a dose dependent pro-inflammatory response one day after a five day exposure, which had declined to control levels after three weeks, suggesting that while the particles induced inflammation, the effect was not persistent. Molecular evidence of an initial pro-inflammatory response followed by recovery was confirmed by genomics and PCR. Modifications of the CuO were conducted in order to improve stability and potentially reduce toxicity. Addition of ascorbate (an antioxidant found naturally in the lung) significantly decreased the toxicity of the CuO *in vitro* as well as the inflammation induced *in vivo*. While other coatings (e.g. PEI) slightly increased toxicity *in vitro* and did not prevent inflammation *in vivo*. The strategy employed was therefore evidence based, allowed refinement of testing to reduce animal numbers required and provided useful data to support safety-by-design strategies.

## JUSTIFICATION OF SAFER-BY-DESIGN SOLUTIONS FOR CuO-NpS: A CASE STUDY

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Understanding the mechanisms of nanomaterials (NMs) interactions with living systems and the environment is a fundamental key to assess and manage NMs potential risk [A. Nel, et al. Science 311 (2006) 622–627]. Establishing correlations between intrinsic physicochemical properties and main properties driving an (eco) tox positive response requires the colloidal characterization of nanomaterials both in water and in (eco) tox relevant media in order to better define their biological identity.

CuO commercial nanopowder (PlasmaChem GmbH), used as active component for antimicrobial wood painting, has been modified within the EU-FP7 SUN project by means of a Safety by (molecular) Design approach (SbyD), in order to produce a material with mitigated (eco) toxicity while preserving performance.

On CuO, four organic modifiers bearing different surface charge were used: positively charged polyethylenimine (PEI), negatively charged sodium citrate (CIT) and sodium ascorbate (ASC), and neutral polyvinylpyrrolidone (PVP). As a general approach, the effect of applied modifications is evaluated in tiers of increasing complexity, aiming to verify whether the modifications induce effective differences with respect to the pristine material, and whether such differences went in the direction of mitigating risk-determinant properties (physicochemical properties, in vitro end-points, in vivo effects) while still delivering the required technological performance. In this view, the research activity was strongly focused on the physicochemical characterization of the modified materials, not only when dispersed in buffered water, but also considering real dispersing media typical of human and ecotoxicological tests. Therefore, CuO was subjected to a complete morphological and colloidal characterization in terms of particle size, hydrodynamic diameter, Z potential, ion dissolution and stability over time, in four different relevant human media and three ecotox media. In particular, a great effort was placed in the assessment of Cu ion release in several media, even developing a new electrochemical platform able to make in situ ion concentration measurements. Furthermore a thermogravimetric study on the organic coatings was carried out, aiming to verify the adhesion of the organic layer, and quantify the amount of organic adsorbed around the particles and the coating excess.

All these results contributed to address the activity toward a more effective matching between biological and physicochemical data. Actually, for CuO the ascorbate coating resulted to be the most promising solution, because its physicochemical properties were modified, with significant positive biological effects in terms of reduced in vitro and in vivo (eco) toxicity.



## Safer nano-product and processes by design

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Safe nanomanufacturing is of crucial importance for sustainable nanotechnology, but unique properties of nanomaterials and their fate and transport require new risk management solution starting from the design stage up to the nanowaste identification and treatment. The SUN WP7 developed new analytical, processing and design TOOLS for safer nanomanufacturing implementable through the nano-product life-cycle:

1) Nano-process analytical technology: enhancing modelling and measurement techniques based on information on nanomaterial properties and associated identified risks. Specifically, a nano-specific process analytical technology (PAT) has been developed to measure and understand in real-time evolution of the product quality parameters and process conditions.

2) Nano-waste treatment: assessing the waste material fractions expected to carry engineered nanomaterials (ENMs), the most prominent ENMs types, and the waste technologies involved in nanowaste handling. Specifically, series of roadmaps for assessing release and exposure from different waste technologies were developed suggesting guidelines for safe handling of nanowaste

3) Safer by product design: controlling nano potential risk by establishing correlations between ENPs design properties (intrinsic, e.g. surface coating), their evolution in testing media (extrinsic properties e.g. zeta potential and colloidal stability) and properties affecting recognised mode of action (risk determinant properties, e.g. ions dissolution). Specifically, different safer by design (SbyD) solutions were proposed and applied at wet and dry state. A tiered approach for their evaluation was proposed in order to identify guidelines for a safer design of nanoprodukt.

The present work outlines results achieved by WP7 (Safe production, handling and disposal) SUN project and how the developed safer nanomanufacturing tools can be integrated in the more general SUN decision support systems (SUNDS).



## ELECTROSPRAY DEPOSITION SYSTEM AS SAMPLE PREPARATION TECHNIQUE FOR NANOPARTICLE ANALYSIS BY ELECTRON MICROSCOPY

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Nanomaterial characterization is a challenging subject due to limitations in analysis method and representativity of sampling procedures.

Although there are many experimental techniques for measuring particle sizes and size distributions, electron microscopy (EM including TEM and SEM) is still considered as the gold standard in this field, especially, when it comes to particles in the nano range (1 nm – 100 nm). The representativity of the particles as sampled on the substrate and their homogeneous spatial distribution must be ensured, to avoid operator bias when selecting the imaged area. Furthermore, agglomeration should be avoided as far as possible. The mentioned problems have not been solved completely by the existing sample preparation techniques. Depending on material the alteration of the true particle size distribution may be unacceptable. One possibility to overcome this problem is the use of an Electrospray System (ES), where the suspension of particles sprayed onto the substrate as charged droplets eases the drying of the solvent and minimizes the agglomeration y electrostatic repulsion. Additionally, the charging of the particles maximizes the collection onto the EM grid that acts as counter-electrode.

The authors have tested the prototype of an ES developed by the company RAMEM under its trademark IONER ([www.ioner.eu](http://www.ioner.eu)). Although electrospray theory is well known and is quite an established technique in many areas of research, no dedicated commercial instruments are available for the preparation of TEM grids yet, so far only electrostatic deposition of aerosols on TEM grids being reported [1].

In this work, the performance of ES as sampling system of EM is assessed by using various particle suspensions. The resulting particle size distributions were compared to more traditional sample preparation strategies like the “drop on grid” method. Operation parameters of the used ES have been optimized in dependence on material. It was found that the particles deposited by electrospray generally show a much more homogeneous spatial distribution on the substrate and the number of single particles increases substantially. The latter finding is much better suited to an automatic image evaluation procedure than the agglomerated particles observed otherwise. The applicability of the technique to a broad range of materials is illustrated by various examples, being able to demonstrate that electrospray deposition of particle

suspensions on a TEM grid is a very promising option for achieving representative particle size distributions [2,3].

[1] M Fierz et al, *Aerosol Science and Technology*, 41 (2007), p. 520.

[2] J Mielke et al, submitted for publication.

[3] The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 604347. Dr. K Löschner and Dr. M Correia (Technical University of Denmark) are thanked for their help to optimize the material dispersion.

#1625

**APPLYING ECHA RECCOMENDATIONS ON GROUPING AND READ-ACROSS OF NANOMATERIALS TO PREDICT IN VITRO GENOTOXICITY (COMET ASSAY) OF TITANIUM DIOXIDE (TiO<sub>2</sub>)**

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Read-across is the preferred method used by REACH registrants as alternative to chemical testing. It involves the formation of groups of similar substances (analogues) from which to infer the biological activity of the target. This is not an easy task in the case of nanomaterials (NMs) because the relation between physicochemical properties of NMs and the corresponding hazard is not fully understood. Several frameworks for grouping NMs have been published (e.g. see Ref. 1–4) but very few examples of their application<sup>5</sup> or of read-across for NMs are available<sup>6</sup>.

We present a practical example of the application of ECHA's recommendations on grouping of NMs<sup>7</sup> for the prediction of in vitro genotoxicity of TiO<sub>2</sub> (Comet assay) by using read-across. We have used publicly available sources of data (OECD dossiers, REACH registrations, SCCS evaluations, and NANOGENOTOX reports) to build a dataset with more than 150 properties for 6 TiO<sub>2</sub> analogues with different sizes, coatings, and crystalline structure. Since the mechanism of genotoxicity of TiO<sub>2</sub> is not fully understood<sup>8,9</sup> we have used chemoinformatic tools such as hierarchical clustering, principal component analysis, and variable selection algorithms to determine the physicochemical properties that can be used to define similar NMs and to elucidate a genotoxicity mechanism that can be used to define a grouping hypothesis. In addition, the applicability of the ECHA Read-Across Assessment Framework(RAAF) to the case of NM read-across has been assessed in the current case study.

*Acknowledgement: The NanoComput project is funded by DG Enterprise under the terms of an Administrative Arrangement between DG Enterprise and the Joint Research Centre.*

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**#1638**

## **Cell based biosensor approach to characterize nanomaterial- cell surface receptor interactions**

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Despite the rapid increase of the understanding of the bio-nano interface *in vitro*, its behaviour in biologically relevant environment remains obscure. Implementation of nanotechnologies both in medicine and other consumer products is currently hampered by the lack of understanding of the mechanism of nanomaterial interactions with living systems. A new label free cell based platform has been developed for the characterisation of interactions between bio-nano interface and its cell binding partners. An optimised procedure for nanomaterial preparation for biosensor analysis has been established. The results obtained in this study signifies the importance of corona proteins in nanomaterial-cell surface interactions. The platform allows studying the effect of surface modifications of nanomaterial on cell surface interactions. The results obtained in the study demonstrate the potential of this platform for the characterisation of bio-nano interface and its binding partners in physiologically relevant environment. This real time, label free biosensor approach may facilitate the understanding of mechanisms involved in nanomaterial binding to cell surfaces, identifying the binding partners of bio-nano interface.

## Surface ligands and functional groups on the surfaces of nanomaterials as determinants of their intracellular uptake and toxicity: A consideration for the grouping of nanomaterials

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A prerequisite for every possible application of nanomaterials is their proper surface functionalization. Surfaces of nanoparticles are in direct contact with biological fluids and therefore the chemical composition and surface activity will largely define their interactions with these environments. As a result, surface properties of nanoparticles are engineered to: Increase their hydrophilicity, prevent their agglomeration, improve their intracellular penetration, increase blood circulation, enhance absorption, improve their pharmacokinetic properties into selected tissues and increase their clinical efficacy. However, most importantly, surface properties of nanoparticles are changed to decrease their toxicity. Attempts have therefore been intensified in the literature in altering the surface properties *via* number of methodologies including surface coating with polymers and/or surface derivatization with functional groups.

Experiments were conducted on number of metal- and carbon- based nanomaterials as well as metal oxides with different ligand carrying functional groups. Results showed that:

1. In the case of those that release ions with high dissolution rates: Functional groups have no effect on their toxicity or uptake as those with and without functional groups all enter the cells and also show high toxicity.
2. For carbon-based, metal-based and metal oxides:
  - a. Presence of COOH and NH<sub>2</sub> functional groups generally increases their uptake and also their toxicity.
  - b. Presence of PEG generally decreases their uptake and also decreases their toxicity.
  - c. Naked nanomaterials depending on their type and shape, generally show high toxicity with high uptake and those that do not enter the cells show lower toxicity.

Since all the tested nanomaterials were negatively charged, our results with cellular uptake could be explained by the type and the organization of the ligands on the surface of the nanoparticles and density rather than the charge of the ligands. On the other hand, our results with the toxicity of ligands could be explained by the functional groups on these ligands. It may therefore be possible to consider intracellular uptake in relation to the surface ligands/functional groups in the grouping of nanomaterials for the purpose of their hazard characterization.

# MODIFYING THE MINIMUM INHIBITION CONCENTRATION (MIC) ASSAY FROM PHARMACEUTICAL TESTING TO A METHOD FOR SCREENING BACTERIAL TOXICITY OF ENGINEERED NANOMATERIALS, IN COMBINATION WITH A SYSTEMS BIOLOGY APPROACH

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Engineered nanomaterials (ENMs) are designed with specific physico-chemical properties in order to satisfy required industrial, medical or commercial uses. Due to the inevitable release of ENMs to the environment there are concerns that these materials will negatively affect microbes that inhabit end-of-pipe ecosystems, such as soils. In this regard, a fast, yet sensitive test is needed to screen the toxicity of a wide range of ENMs against bacteria. This work assessed the effects of coated and uncoated ENMs on *Escherichia coli* K-12 MG1655 by using a consistent method that was reproducible. Global RNA sequencing was also used to describe gene expression levels, via messenger RNA abundance, in response to different test exposures.

The minimum inhibition concentration (MIC) assay which quantifies complete growth inhibition in bacteria was adapted to work with ENMs and used to rank the toxicity of nanodiamonds, cadmium telluride (CdTe) quantum dots, spherical and tubular titanium dioxide (TiO<sub>2</sub>), multi-walled carbon nanotubes (MWCNT), silver (Ag) and cupric oxide (CuO). Bulk (microscale) material counterparts and metal salts were also tested. Mercuric chloride was used as the positive control for zero growth. Bacteria were exposed for 12 h at 37 °C to a dilution series of the test suspensions, with nominal concentrations from 1.5 to 100 mg l<sup>-1</sup> in 96-well plates (*n* = 6 plates/treatment). All test suspensions were characterised for particle number concentration and particle size distribution. Metal concentrations and dissolution rates were also measured. Growth of *E. coli* K-12 was determined by measurements of optical density (*OD*<sub>440</sub>) and glucose consumption.

The smallest concentration, per unit mass, at which complete growth inhibition occurred (i.e., MIC) was used to rank ENMs toxicity, as follows: CdTe quantum dots ammonium-coated (6 mg l<sup>-1</sup>) > Ag uncoated nanoparticles (12 mg l<sup>-1</sup>) > CdTe quantum dots carboxylate-coated (25 mg l<sup>-1</sup>) > Ag microscale uncoated (50 mg l<sup>-1</sup>) > CdTe quantum dots polyethylene glycol-coated (100 mg l<sup>-1</sup>). No MIC values were recorded for ENMs of CuO, TiO<sub>2</sub>, MWCNT and nanodiamonds. Silver nitrate was consistently toxic, as mercuric chloride, at all the tested concentrations. The metal salt and bulk equivalent to the quantum dots were not toxic to *E. coli* K-12. All copper-based materials, displayed a growth stimulatory effect with increasing test concentration, up to 50 mg l<sup>-1</sup>; and only copper sulfate displayed more than 50 % growth inhibition at 100 mg l<sup>-1</sup>. Between CuO bulk and uncoated CuO nanoparticles there was no growth difference at all tested concentrations (ANOVA, *p* > 0.05).

As a screening tool, the modified MIC assay was found to be sensitive enough to rank ENMs according to the level of hazard. On-going gene expression profiling is also seeking to provide a more in-depth insight into the mechanisms of ENMs toxicity in *E. coli* K-12.

The research was funded by EU FP7 NANOSOLUTIONS Project, Grant Agreement No. 309329.

**NOVEL HIGH RESOLUTION DIFFERENTIAL MOBILITY ANALYSER (HRDMA) FOR SIZE CHARACTERIZATION OF NANOPARTICLES SMALLER THAN 5NM**

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Nanomaterial characterization is a challenging subject due to limitations in analysis method and also in sampling. Aerosol technology has been detecting and characterizing nanoparticles for long time and some of their developments can be of use in nanotechnology.

Having accepted and validated methods to determine several characteristics and descriptors of NanoParticles (NPs) is an open challenge, being size one of them. Since the nanoparticle range, as the European Commission's definition recommends (Recommendation 2011/696/EU), stands between 1 and 100 nm, very different properties are found in this huge range of sizes, therefore different methods will be required for the extreme ranges. It is accepted that NP size affects tremendously its properties, toxicity among them. There is an added difficulty since no on line standard method is accepted for the size determination of the smallest range in nanoparticles, despite Electron Microscopy (EM) methods are the most accepted ones.

Cylindrical Differential Mobility Analysers (DMAs) are used for determining the size distribution of bigger NPs. However, for the smallest ranges, technical challenges are found due to the high mobility of the smallest charged NPs that drives to higher losses and low sensitivity. Additionally, the resolution (ability to distinguish NPs of similar sizes) of the cylindrical DMAs has limits with the smallest NP sizes. To solve the technological challenges of the DMAs for the smallest NPs, RAMEM has developed a parallel plate DMA in which the NPs follows shorter and simpler trajectories, resulting in higher transmission, minimum losses and high sensitivity. The instrumental design has allowed a resolution 4 times higher than the classical cylindrical DMAs.

The inversion between mobility and size is needed because with the smallest NPs, the charging stage is an open challenge. With the smallest NPs the charging efficiency is very low and some of the charged particles have multiple charges. Having a high yield of charged NP with only one charge avoiding radioactive charger and neutralizers is a challenge for this size range. There are neutralizers in the market but their performance for the smallest NPs is still open. RAMEM is investigating different alternatives in order to achieve a well know equilibrium charge distribution in the smallest nanoparticles to univocally link the mobility with the size.

RAMEM will present results of DMA characterization: Resolution and Transmission for the smaller NPs range using mobility. Some neutralization experiments results will also be shown for the NPs in the smaller range. The successful completion of the neutralizer will allow the size classification of the smaller NPs, launching an instrument that fills the gap in size characterization for NPs smaller than 7nm. The resulting instrument will be able to distinguish NPs with differences of 2% for the smallest NPs.



## THE IMPACT OF ENGINEERED NANOPARTICLES ON AQUATIC SPECIES: ISA-TAB COMPATIBLE DATASET MINED FROM LITERATURE (2007-2016)

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The detailed characterisation of engineered nanoparticles (NPs) is highly significant as it can provide detailed information about their physicochemical properties and more importantly their effects (e.g. toxicity) on the environment and living organisms. The large amount of potential characterisation parameters (e.g. 36 endpoints listed in OCED Workshop on physical-chemical characterisation of nanomaterials) in combination with a lack of total understanding of which parameters cause toxicity, has led scientists to follow different characterisation routes, which results in a discontinuity in research and difficulties in comparability of the, so far, published results. As a result, the need for a more standardised characterisation protocol is needed, which will offer the needed continuity and comparability and will allow for safer conclusions of the causes of NP toxicity to be reached. For these reasons, among others, a database (DB) was created as part of the EU FP7 ModNanoTox project, which contained extracted data from published studies dealing with the toxicity of engineered NPs on aquatic species, with inclusion criteria based on the study meeting minimum characterisation requirements.

The extracted data was then graded and categorised depending on the degree of detailed analysis performed over time and analysed using non-linear statistics (e.g. CATPCA) so as to identify the characterisation parameters which are considered more significant by researchers and are thus included in more studies and analysed in higher detail (e.g. in exposure medium, more time points) over time, as well as to identify whether those parameters are also those who are correlated more highly with observed NP toxicity relative to other endpoints present in the DB. The results suggest that the NP parameters which are studied in more detail over time (across literature from 2007-2016) are morphology, hydrodynamic diameter, concentration, aggregation and dissolution, which, so far, account for 28.2%, 54.7%, 15.9%, 22.9% and 40.0% respectively of the total DB entries. Changes in any of these parameters during an exposure test can lead to a completely different interaction of the NPs with the surrounding media and the environment in general, as modelling, performed as part of the ModNanoTox project, also suggests. At the same time, analysis of the dataset demonstrated the need for detailed laboratory characterisation of commercially available NPs since significant differences were, in some cases, found between the provided specification sheets and the actually measured characterisation parameters. For example, the average % difference of NP diameter between the commercially provided and laboratory values was 164.00% and ranged from 19% to 1727%.

## EXPLORING CORRELATION PATTERNS ON TOXICITY OMICS DATA

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High-throughput experimental methods such as RNA deep sequencing transcriptomic approaches, oligonucleotide microarrays and mass spectrometry (MS) experiments, are becoming increasingly popular and their usefulness has been demonstrated on nanoparticle (NP) data [1,2]. Recently, many studies have demonstrated that by integrating omics data with genomic knowledge to construct pre-defined features, results in higher performance in predicting clinical outcomes or profiles and higher consistency between the results of different studies [3]. In this work we study the correlation structures in omics and physicochemical NPs data, as well as their predictive ability using read across modelling. Specifically for the omics data, enrichment analysis is considered as means of filtering the data prior to prediction. Our method was applied to publicly available cancer data [2,4] reporting significantly high accuracy values depending on the filtering of the data, the number of neighbours or the distance metric considered. Furthermore we showed that the use of constitutional genetic variation for predicting toxicity response increases performance considerably.

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## IMPLEMENTATION OF READ-ACROSS BETWEEN (NANO)MATERIALS FOR HAZARD ASSESSMENT IN GUIDENANO

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For the GUIDEnano risk assessment tool for (nano)materials (NMs), a practical read-across approach is developed to help assess the similarity between exposure relevant NMs and NMs that have been used in inhalation toxicity studies.

The similarity assessment involves algorithms allowing the comparison of NMs based on the following physicochemical properties: chemical composition, dissolution, shape and primary and aggregated (hydrodynamic or aerodynamic) size distributions.

Currently only comparisons between uncoated NMs of the same chemical composition are possible, because for coated NMs information on the chemical composition, thickness and stability of the coating would be required, which will generally not be available. For uncoated materials, depending on the chemical composition, crystallinity and purity will also be taken into account.

Dissolution rates of NMs in comparable environments (e.g. inhalation exposure scenarios) will mostly depend on size and chemical composition. Since we only consider analogous chemical compositions, and similarity of size distributions is assessed separately, dissolution properties are only used to identify highly soluble NMs, for which the size and shape similarity assessment is considered irrelevant. For other materials, similarity of shape is assessed by rules including the aspect ratio and rigidity.

Two materials with different aerodynamic size distributions will have different deposition profiles in the lung, which the tool will take into account by including a simplified lung-deposition estimation.

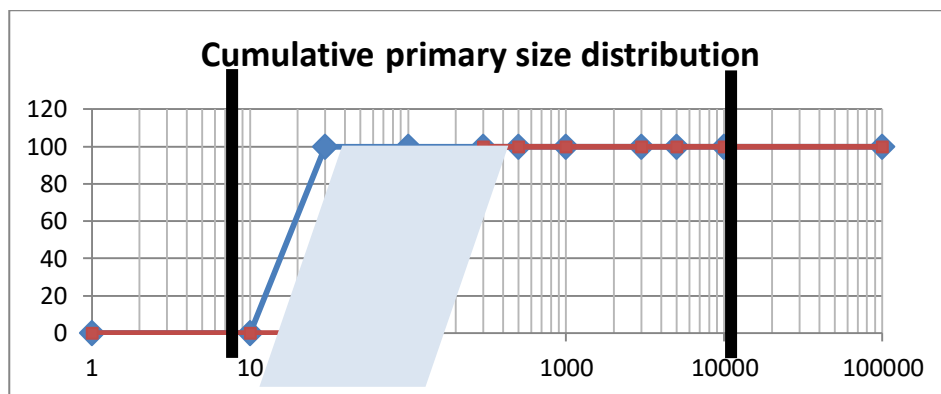


Figure 1 Cumulative size distribution curves for a fictive exposure relevant material M1 (red) and a studied material M2 (blue). The shaded area depicts the difference in surface areas under the curves in the comparison of their primary size distributions. In this case, the similarity score is 0.7.

Next to aerodynamic size related lung deposition, two materials may exert different effects in the lung depending on their primary size distributions, due to differences in cellular uptake, clearance rates and reactive surface areas of (potentially disaggregated) particles. Therefore, the tool will compare the primary size distributions of two materials in the alveolar fraction (between 10 and 10000nm), by comparing the surface area under their cumulative size distribution curves (Fig 1).

The similarity score will be defined as  $S = 1 - (\text{difference in areas under the curve} / \text{total area in the size range considered})$ , resulting in a value ranging from 0 to 1 (none to maximum similarity).

Only studies with NMs that have a similarity score above a defined threshold for the relevant physicochemical properties will be considered acceptable for the hazard assessment. Embedding these types of rules in the GUIDEnano tool enables a quantitative, transparent and uniform selection of studies that have used a material with similar hazard-relevant properties compared to the exposure relevant nanomaterial. In addition, the overall similarity assessment score can be used to calculate an associated uncertainty factor in the risk assessment. (Funded by Grant Agreement No.604387).

## DESCRIBING NANOMATERIALS ACCURATELY IN SUPPORT OF CATEGORIZATION AND READ-ACROSS

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The variety of types, diversity, and complexity of nanomaterials offer many opportunities to create nanomaterials with specific functionality in medicine, food, cosmetic products, and consumer goods. At the same time, the large number of nanomaterials makes it virtually impossible to test all possible compositions, shapes, sizes, and other characteristics. Consequently the accurate prediction of functionality is increasingly important in determining both potential benefits and risks of new and proposed nanomaterials. Success in prediction is critically dependent on identifying specific features of a nanomaterial that determine its behaviours and functionalities; once identified those features can be used to predict behaviours and functionalities of other nanomaterials with similar features. The process of feature identification is complicated by the surface reactivity of nanoparticles, especially in biological and environmental media. The CODATA Uniform Description System for Materials on the Nanoscale (UDS) has been developed to define a consistent and comprehensive method for describing virtually every type of feature found in a nanomaterial. For individual nano-objects, these features include chemical composition, crystallographic and physical structure, shape, size, surface characteristics, specifications, and production (processing). For collections of nano-objects, the information includes composition, size distribution, physical structure, association, interfaces, surface features, and topology, as well as production. In our presentation, we discuss how the UDS can be applied to categorization and read-across activities, with special emphasis on using the UDS to identify features that control biological activity. We also discuss how to describe a nanomaterial throughout its life history, from a pristine (as manufactured) state to an actual test situation. We demonstrate how the UDS can improve reporting nanomaterials test results thereby leading to more accurate functionality prediction by read-across-methods. Explore the relationship of the UDS to nanomaterials ontologies, international test standards, and QSAR (quantitative structure-activity relationship) models.

### Exposure assessment along the life cycle of nano-enable products

**# 1516**

#### **Dermal and dermal-to-oral exposure to nanomaterials**

Araceli Sánchez Jiménez, Anne Sleenwenhoek, Aiga Mackevica, Mikael Emil Olsson, Steffen Foss Hansen

Nano-scale materials have many industrial applications. However, exposure to these materials may pose a risk to human health. Penetration of nanoparticles through the skin is probably quite limited, but there is greater possibility for inadvertent ingestion from hand-to-mouth contacts. However, little is known about the transfer efficiencies from surfaces-to-skin or from skin-to-skin of nanoparticles. This presentation will present preliminary results from The SUN project.

The transfer of SiO<sub>2</sub> powder from skin (finger) to the perioral area was investigated using wipe sampling method. TEs were around 100% and were significantly affected by the loading on the finger and the TEWL (Trans Epidermal Water Loss) of the perioral area.

The potential for dermal transfer from CuO treated wood (acrylic paint) was tested by surface wiping (textile wipes were used as a surrogate for skin) followed by particle extraction from wipes by ultrasonication and analysis using single particle ICP-MS. Wiping tests were conducted on freshly painted wood and sanded wood (to simulate accelerated weathering conditions and wear-and-tear).

The results showed that there is nearly no CuO release from the painted surface when the paint is fresh. However, release from sanded painted surface was considerably increased, resulting in transfer of up to  $5 \times 10^5$  CuO particles per cm<sup>2</sup> (1.2 ng CuO/cm<sup>2</sup>) whereas for paint without sanding it was around  $2 \times 10^5$  particles per cm<sup>2</sup> (0.4 ng CuO/cm<sup>2</sup>). The mean sizes of the released particles were around 84 nm and 79 nm for CuO-paint without and with sanding, respectively.

Further work is needed to refine the test procedures and to conclusively determine whether nanoparticles are transferred to the skin more readily than micron particles and the factors affecting the transfer.

The authors acknowledge the EU Commission FP7 program for funding the SUN project (Grant agreement No: 604305)

## INVESTIGATION OF THE DIETARY BIOAVAILABILITY OF SILVER NANOPARTICLES IN RAINBOW TROUT USING AN EX VIVO GUT SAC TECHNIQUE

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The dietary bioavailability of nanoparticles (NPs) has received little attention in fishes. Here we investigated the dietary bioavailability of silver NPs (Ag NPs) in rainbow trout using an ex vivo gut sac technique that enables the bioaccumulation potential to be screened. Adult rainbow trout ( $140 \pm 28$  g) were euthanised and the whole gastrointestinal tract was removed and rinsed through with physiological saline (PS) to remove food debris and faeces. Individual gut compartments (stomach, anterior [including pyloric caeca], mid and hind intestine) were filled with either PS (control), 1mg/L Ag (as AgNO<sub>3</sub>) or 1 mg/L Ag NPs. Both ends of the tissue were tied using suture thread and then individually incubated in PS for 4 h at 15°C. At the end of the exposure period, the gut sacs were removed from the tubes and the tissues were opened and the contents (the non-tissue associated fraction) drained to waste. The tissue was then rinsed with PS containing EDTA to chelate and displace loosely associated silver ions and NPs. The stomach, mid and hind intestine tissues were deconstructed into the enterocytes and muscularis. Pyloric regions were separated into the whole anterior intestine (enterocyte and muscularis) and caeca. Samples of the serosal PS were also collected to measure uptake of the materials into the blood compartment. Tissues were freeze dried, digested with nitric acid (70°C for 4 h) and total Ag concentrations measured using ICP-MS. Small tissue snips of the mid and hind intestine were taken for histological analysis to assess tissue integrity. In addition, tissue samples from the pyloric caeca, mid and hind intestine were taken for analyses of lipid peroxidation (TBARS) and total glutathione (GSH) concentration. Histological observations confirmed tissue integrity was maintained throughout the 4 h incubation. TBARS and GSH concentrations were also unaffected by exposure to AgNO<sub>3</sub> and Ag NPs. Silver concentrations were elevated in the enterocyte layer in the stomach, mid and hind intestine after exposure to AgNO<sub>3</sub> or Ag NPs and compared to controls but there was no significant difference between the two materials. Overall, data indicate highest uptake/association of AgNO<sub>3</sub> and Ag NPs is in the hind intestine. Data also indicate that gut sacs are an effective tool for assessing dietary bioavailability of NPs in fish in the first instance but their use should be verified with an in vivo dietary trial with fish where required.

**NANOCOMPOSITES END-OF-LIFE STUDIES: A WAY TO IMPROVE THEIR SAFE BY  
DESIGN BEHAVIOR WITHIN THE FRAME OF THE GUIDENANO  
EUROPEAN PROJECT**

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For more than one decade, the increase of nanoparticles (NPs) used in industrial products and the uncertainty on their toxicity induced a new way of taking control of the development: the safe by design approach combining nanocomposite properties with its end-of-life behavior. The main goal is to choose the formulation with less NPs released when the nanocomposite becomes a waste. In the frame of Guidenano European project, we investigated the nanocomposites high thermal degradation and the released NPs potential in the combustion fumes.

For this purpose, we developed a high temperature furnace to study the thermal degradation of polymers containing nanoparticles, in agreement with the European parliament directive (2010/75/EU – art. 50). Several equipment were connected to the furnace exit to measure and observe the aerosols generated by the process. The incineration protocol developed in our laboratory showed stable and reproducible results.

In this lecture, we focused on the results obtained during the thermal degradation of two different kind of nanocomposites: PolyPropylene (PP) loaded with TiO<sub>2</sub> NPs and PolyAmide (PA 66) loaded with MWCNT. The PP incineration shown that the aerosols generated during incineration are mainly composed of NPs. The addition of TiO<sub>2</sub> NPs in PP induced a slight increase of the amount of released NPs and slightly modified the PP thermal degradation kinetic, Figure 1-A. In parallel, the microscopy/chemical analysis (SEM/EDS) observations showed that aerosols were composed of carbonated aggregates/agglomerates NPs coming from the PP matrices degradation, and the final ashes were mainly composed of TiO<sub>2</sub> aggregates/agglomerates NPs, Figure 1-B. In parallel, the PA incineration behavior was analyzed to improve the incineration protocol. The aims are to highlight the versatility of the technic and also to give predictive trends on the nanocomposites incineration behavior.



The combination of all the results will provide the industrial partners the best formulation for tomorrow's safe-by-design nanocomposites.

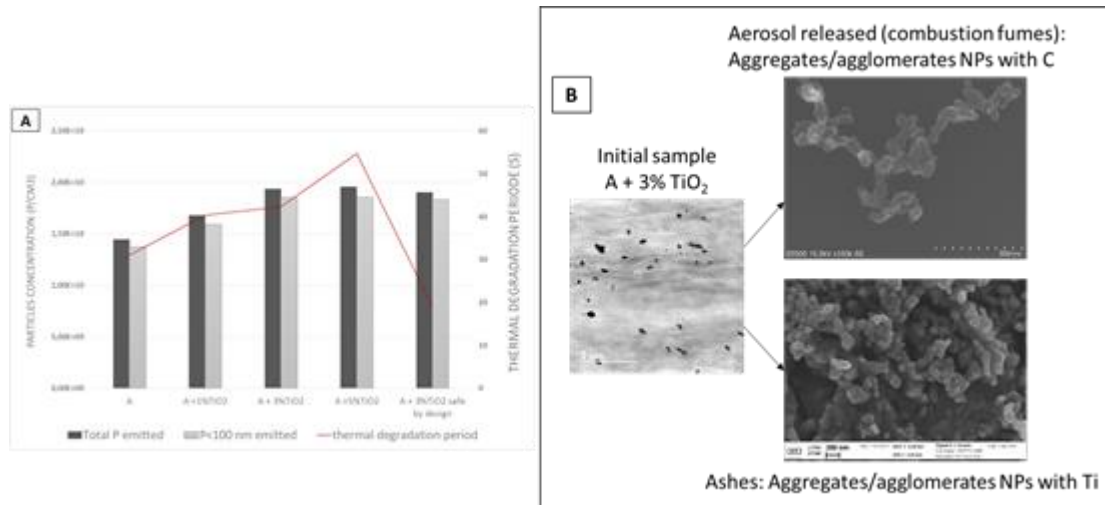


Figure 1: Impact of TiO<sub>2</sub> NPs loaded in PP matrices on NPs emission and on thermal degradation period (A); SEM/EDS observation before and after thermal degradation (B)

## Effects of medium composition on the aquatic fate of copper nanoparticles

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Given the widespread manufacture and application of Cu nanoparticles (CuNPs), there is a necessity to evaluate their fate in the aquatic environment at field relevant conditions. In this work, the effects of aqueous parameters (pH, divalent cations and dissolved organic carbon (DOC) concentrations) on the fate of CuNPs and Carbon coated CuNPs were systematically investigated. The results show that in the absence of DOC, the aggregation was enhanced with addition of cations, while pH in the range of 6-9 did not significantly affect aggregation; the dissolution was highly affected by pH and cations had a minor effect on dissolution. In the presence of DOC, the solubility of CuNPs was significantly decreased and the stability of CuNPs was enhanced at low cation concentrations ( $\leq 2.5$  mM), whereas at high cation concentration (10 mM), the aggregation was enhanced, resulting in an intensive sedimentation. With regard to the carbon surface coating, it mitigated the aggregation of CuNPs in the absence of DOC and divalent cations. However, in the presence of DOC and divalent cations, the aggregation trends for both the CuNPs and C-CuNPs were similar, indicating that the aquatic parameters (i.e. NOM and divalent cations) are more important to determine the aggregation of CuNPs, relative to the initial carbon surface coating. In addition, it moderately inhibited the solubility of CuNPs, compared to the uncoated CuNPs.

Overall, our results demonstrate that the fate of CuNPs is highly determined by environmental factors, especially DOC, rather than by the initial carbon surface coating.

## ***In vitro* exposure of ZnO nanoparticles and Mesoporous Silica nanoparticles on immune and male germ cells**

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Nanotechnology is a science which has caught the fancy of one and all. Materials reduced to the nanoscale show some novel properties which are different than their bulk material counterparts due to their small size and large surface area to volume ratio. Increasing use of nanomaterials (NMs) has raised concerns about their potential risks to human and other life forms. ZnO nanoparticle (ZNP) and mesoporous silica nanoparticles (MSN) are amongst important materials having profound applications in various fields. Hence under the present study, *in vitro* effect of different concentration of ZNP and MSN on two different and unique models viz ; neutrophils and male germ cell were assessed in terms of cytotoxicity test (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NR: Neutral red assay) as well as major anti-oxidative enzyme measurement (SOD: Superoxide dismutase, GPX: Glutathione peroxidase). Red blood cell (RBC) hemolysis assay was done in order to compare the relative toxicity of the two nanoparticles. Treatment of neutrophils and germ cell with 50µg/ml and above of ZNP resulted in a significant ( $p<0.01$ ) decrease in percentage viability (%viability) compared to control as depicted by MTT and NR assay. Likewise, a significant ( $p<0.001$ ) increase in SOD and GPX activity was observed upto 50µg/ml of ZNP treatment followed by significant reduction ( $p<0.05$ ) in activities at higher concentration. But in case of cells treated with MSN there was no significant changes in %viability as well as in the activities of oxidative stress enzymes even at higher concentrations. LD<sub>50</sub> obtained from percentage hemolysis (%hemolysis) assay of RBCs also confirms that ZNP at higher concentration is toxic as compared to MSN. Additionally, treatment of germ cell with higher conc. of ZNP reveals a significant loss ( $p<0.05$ ) in viability and membrane integrity as seen by Eosin-Nigrosin assay and hypo-osmotic swelling test (HOST), respectively. As suggested by their toxicity assessment, the results placed ZNP at a higher level than MSN. Hence safety assessment of nanoparticles on a range of models is mandatory before their exploitation for various applications.

**#1558**

## **LICARA NANOSCAN - A FIRST TIER TOOL FOR THE SELF-ASSESSMENT OF BENEFITS AND RISKS OF NANOPRODUCTS IN THE SUN DECISION SUPPORT SYSTEM**

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The fast penetration of nanoproducts on the market under conditions of significant uncertainty of their environmental properties and risks to humans creates a need for companies to assess sustainability of their products. Evaluation of the potential benefits and risks to build a coherent story for communication with clients, authorities, consumers, and other stakeholders is getting to be increasingly important, but SMEs often lack the knowledge and expertise to assess risks and communicate them appropriately. This presentation introduces LICARA nanoSCAN, a modular web based tool that supports SMEs in assessing benefits and risks associated with new or existing nanoproducts. This tool is unique because it is scanning both the benefits and risks over the nanoproducts life cycle in comparison to a reference product with a similar functionality in order to enable the development of sustainable and competitive nanoproducts. SMEs can use data and expert judgment to answer mainly qualitative and semi-quantitative questions as a part of tool application. Risks to public, workers and consumers are assessed, while the benefits are evaluated for economic, environmental and societal opportunities associated with the product use. The tool provides an easy way to visualize results as well as to identify gaps, missing data and associated uncertainties. The LICARA nanoSCAN has been positively evaluated by several companies and was tested in a number of case studies. The tool helps to develop a consistent and comprehensive argument on the weaknesses and strengths of a nanoproduct that may be valuable for the communication with authorities, clients and among stakeholders in the value chain. LICARA nanoSCAN identifies areas for more detailed assessments, product design improvement or application of risk mitigation measures. LICARA nanoSCAN is currently being implemented as a First Tier assessment in the SUN Decision Support System.



## Conceptual framework on nano benefits and risks

### 0. Nanoproduct and legislation

1. Environmental  
benefits

2. Economic  
benefits

3. Societal  
benefits

4. Public health &  
environmental  
risks of nano

5. Occupational  
health risks of  
nano

6. Consumer  
health risks  
of nano

### 7. Decision support

## EXPOSURE TO CERAMIC NANOPARTICLES DURING PLASMA SPRAYING

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Atmospheric plasma spray deposition is a frequently used technology for producing high-performance surfaces required in industrial processes. It is used to deposit different coatings on surfaces in order to achieve enhanced properties such as wear, corrosion, or heat resistance, while maintaining the structural properties of the underlying material.

Because of its high-energy nature, this industrial process has a major potential for nanoparticle generation and release into workplace air. In the framework of SINN-ERA-NET project CERASAFE, the objectives of this work were to characterise nanoparticle release mechanisms during atmospheric plasma spraying in an industrial pilot plant, and to characterise their impacts on workplace exposure.

Nanoparticle concentrations were characterised by means of simultaneous monitoring inside and outside the plasma spraying chamber using particle counters (DiscMini and CPC), as well as particle number size distribution monitoring outside the chamber (TSI NanoScan). Particle number concentrations (N) were also monitored in outdoor air, to identify potential nanoparticle release to the environment. Samples were collected on TEM grids for morphological characterisation. Spray coating was carried out using different types of feedstock (coating solution), both nano-suspensions and micro-suspensions.

Results evidenced major nanoparticle emissions during plasma spraying. N concentrations (10-700 nm) reached up to  $3.0 \times 10^6 \text{ cm}^{-3}$  with a mean diameter of 36 nm during the spraying process (Fig. 1), in contrast with the initial background concentrations of  $10^4 \text{ cm}^{-3}$  (66 nm in diameter). N decreased to around  $3 \times 10^5 \text{ cm}^{-3}$  after spraying and before opening the door of the chamber, and remained above background concentrations even after the door had been opened. These emission concentrations had a significant impact on worker exposure concentrations (outside the spraying chamber), where N reached  $3 \times 10^5 \text{ cm}^{-3}$  with a mean diameter of 50 nm. As in the case of the projection chamber, the mean particle diameter increased after the end of the projection due to coagulation and condensation processes. Similar patterns were obtained irrespectively of the use of nano- or micro-suspensions as feedstock, evidencing the process-generated nature of the nanoparticles detected.

Mitigation strategies were proposed to minimise worker exposure. Nanoparticle concentrations monitored after their implementation proved to be effective (70% decrease in N). This work evidences the relevance of process-generated emissions with regard to workplace exposure to nanoparticles, and the need for real-world assessments.

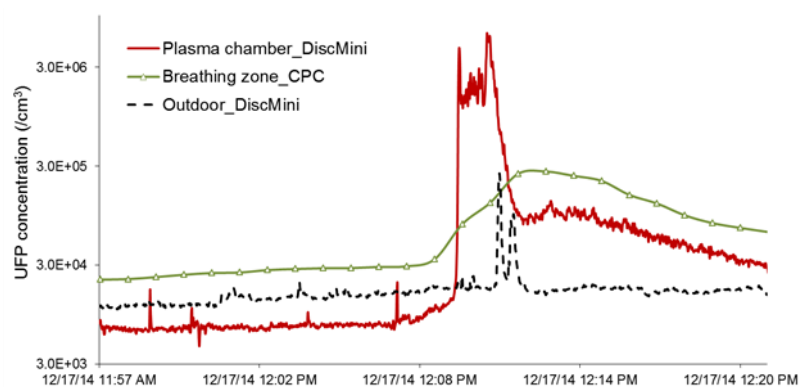


Figure 1. Ultrafine particle (UFP, 10-700 nm) concentrations monitored at three locations in a real-world industrial setting.

This work was supported by the Spanish MINECO through project PCIN-2015-173-C02-01, under the frame of SIINN, the ERA-NET for a Safe Implementation of Innovative Nanoscience and Nanotechnology, by SIINN-ERANET project CERASAFE (id.:16).

## RELEASE OF NANOMATERIALS: NANOMATERIAL INTEGRATED IN CAR BUMPERS

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There is still limited knowledge about the extent of particle release and the characteristics of the dust particle generated during realistic use and mechanical reduction of matrix nanocomposites. One of the key questions is whether there are circumstances during which nanomaterials can be released and result in the exposure to free or accessible nano-objects and their aggregates and agglomerates (NOAA) during such processes. Within the Sustainable nanotechnology (SUN) project we studied the potential release (both on type and quantity) of NOAA and other (formulated) nanosized particles during sawing and drilling in two different car bumpers, which only differ in the presents of carbon nanotubes (CNT) or organic pigment.

In a chamber study (19.5 m<sup>3</sup>) 36 experiments were performed with variation in the abrasive activities (drilling or sawing), different nanomaterials in car bumpers (CNT or organic pigment), the instrument settings during the abrasive activities (speed tool or total treated surface area), the ventilation rate (no ventilation or 3.5 Air changes per hour (ACH)) in the room. In addition, several experiments were performed three times to test the variability in release under similar conditions. Furthermore, during the experiments also dermal deposition was measured in the near-field and far-field of the chamber. Temperature and humidity were measured and controlled (respectively ~21 °C, range 20–22 °C and ~60%, range 52–74%). The selecting mechanical band saw table and drill table were selected based on the type of engine (induction), to ensure no nanoparticle production by itself as is the case for electric engines (which were used in previous abrasive studies). The measurement approach used covers a background measurement (both near field and far field) and measurements during the experiments (both near field and far field), which were performed for 5 minutes. The measurement instruments used were Aerosol Particle Sizer (APS, TSI 3321), Scanning Mobility Particle Sizer (SMPS, TSI 39), Electrical Low Pressure Impactor (ELPI (+), Dekati), DustTrak (TSI, Monitor 8532), Condensation Particle Counter (CPC, TSI 3007), Diffusion Size Classifier miniature (DiscMini) and offline sampling instruments for SEM analyses. Data was analyses using Autoregressive Integrated Moving Average (ARIMA) models in the statistical software R.

Results of the experiments show high peak concentrations during sawing of car bumpers up to 1E6 #/cm<sup>3</sup> while no significant elevations in release were found for drilling. In addition, no differences were observed between both car bumpers in release and mechanical ventilation had a large effect on the concentrations in the room). SEM analyses show melted matrix particles including the CNT and organic pigment nanomaterials for the sawing experiments, most likely explained by the mechanical sawing instrument which produces heat at the sawing surface. No free NOAA were observed. Regarding the dermal deposition no



NOAA were detected. As analyses are ongoing, more detailed results will be provided during the presentation.

The authors would like to acknowledge the EU Commission FP7 program for funding this project (Grant agreement No: 604305).

## DERMAL MODEL FOR NANOMATERIALS

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Recent health related nanoparticle research has mainly focused on biological activity, toxicity and (occupational) exposure through inhalation. However, it has been estimated that dermal exposure can be a major route of exposure in specific occupational settings. For bulk compounds multiple models are available to estimate dermal exposure, both in occupational settings and for consumer products. However, current dermal exposure models need to be examined for their reliability and usability in the assessment of nanoparticles, possible nanospecific weaknesses will need to be identified and, when necessary, adaptations will have to be made to eliminate these weaknesses. Within the Sustainable Nanotechnology (SUN) project we have continued the work on the pre-normative research under CEN Mandate/461 Nanotechnologies. We have answered the following questions: 1) What are the unique properties of nanoparticles and which variables are necessary to model them? 2) How do the current dermal models work, are they suitable for nanoparticles and what are their nanospecific weaknesses? 3) What would a nanoparticle-specific model look like and where does it deviate from non-NP models?

In this SUN study, first a systemic literature review was performed, exploring the state of the art in the field of nanoparticles in relation to dermal exposure. Subsequently, a short review was performed on recent studies illuminating the nanospecific properties that affect dermal penetration. Next, a selection of dermal exposure models and their underlying framework were examined. This was followed by an evaluation of their nanospecific weaknesses and strengths using the gained insights into the unique properties of nanoparticles and which of these properties are especially relevant for dermal exposure. The results were discussed with an expert panel in order to develop a concrete (theoretical and not data driven) framework for a nanospecific dermal exposure model.

The physiochemical properties of nanoparticles which are expected to be important based on the review are primary particle size, exposed size distribution, particle number concentrations, particle mobility, specific surface area, surface properties & coating, coagulation, adhesion, cohesion and removal.

The RISKOFDERM dermal exposure model was selected as it is a quantitative based model (based on non-nano data) with a broad applicability domain. Theoretical adaptations were made for the RISKOFDERM model to become nanospecific. In addition, adaptations are general applicable and can also be made for other dermal exposure models. Future research should focus on the calibration and validation of the model.

The authors would like to acknowledge the EU Commission FP7 program for funding this SUN project (Grant agreement No: 604305).

## REPLICATE OF GUIDENANO CASE STUDIES UNDER CONTROLLED CONDITIONS TO SUPPORT DISPERSION MODELS

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Within the GUIDEnano project, a European research project funded under the 7th framework program, the exposure to nano-enabled products of a total of eight case studies from different industry sectors were investigated.

In order to support the emission rate estimation and the dispersion models from real case studies, replicates of these scenarios are being reviewed in an exposure chamber to obtain additional information with controlled conditions and zero background.

Experiments take place in the exposure chamber at ITENE, which has 24 m<sup>3</sup> and a laminar flow of near 2800 m<sup>3</sup>/h which cleans the ambient to below 2 part/cm<sup>3</sup>. In this way, the noise and background from real case studies is removed and the release of particles during the process can be measured accurately.

Processes able to be replicated were evaluated and selected from the measurements in industrial facilities analysed previously. Several factors were considered to select the scenario to be simulated:

- The possibility of replica. When heavy machinery is involved, the essay cannot be replicated in the room with the same characteristics.
- The likelihood of exposure. Scenarios with medium to high probability of exposure are preferred.
- Gaps during the case studies. Processes in which any external factor affected the results (e.g. devices not recording data, neighbour activities that masked the target scenario, etc.) were replicated.
- The available information about the characteristics of the process. It is aimed to achieve the same or similar conditions: ENMs concentrations, energy of the process, RMMs present, etc.

Additionally, some studies were done replicating standard operations involving the handling and manipulation of different powdered nanosubstances (graphene, SiO<sub>2</sub>, TiO<sub>2</sub>) during side processes such as packaging, pouring or weighting.

With the purpose of using the same nanomaterials from the real case studies, manufacturers were contacted. However, in some cases, it was employed too a commercial product with similar characteristics for comparison or blank testing.

The completion of the focused chamber studies aims to provide information to be used in the estimation of emission rates of ENMs from occupational scenarios. The comparison of these results with those of the case studies would help to support the models of emission and dispersion of ENMs.

## A multi approach study of the effect of the exposition to polystyrene nanoparticles on *Daphnia magna*

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Nanoparticles (NPs) are defined as particles that have at least one external dimension between 1 and 100 nm. Because of their unique characteristics - i.e. high surface area to volume ratio- the use of NP in different industrial applications is steadily on the rise, and so it is the risk of their release into the environment<sup>1, 2</sup>. Nevertheless, there is still a lack of knowledge about the implications of NP exposure on ecosystem function and to human health.

The aquatic crustacean *Daphnia magna* has been widely used as an environmental indicator species given its high sensitivity to the presence of toxicants in the water<sup>2, 3</sup> and its transparent exoskeleton. *D. magna* are filter feeders able to ingest particles in the size range of 20–70 µm<sup>2</sup>. Therefore these organisms are an easy entry point for NP to the food webs, causing multi-trophic level effects. Besides, other molecules present in the environment may adsorb to the NP surface, modifying its corona. These interactions are difficult to predict and can lead to an alteration of the toxicity and bio distribution of the NP<sup>3</sup>.

Polystyrene NPs can be used to shed light about the possible environmental effects of both NPs and microplastics. Here, we present a study in which we assess the effects on *D. magna* of an acute exposition to polystyrene NPs, in terms of viability, reproduction, intake and excretion.

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### Acknowledgments

The project leading to this application has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 691095 and from Junta de Castilla y Leon, project No BU079U16.



## DEVELOPMENT OF A REAL-TIME INFORMATION AND MONITORING SYSTEM TO SUPPORT THE RISK ASSESSMENT OF NANOMATERIALS UNDER REACH

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Within the LIFE NanoMONITOR project, an innovative systems to monitor the concentration of ENMs in indoor workplaces and the environment is being developed. This systems is based on the development of an on-line data analysis tool for collecting and archiving data on the environmental concentration of Engineered Nanomaterials (ENMs), coupled with a newly developed prototype and low cost nano-pollution monitoring systems able to continuously measure key airborne nano-pollutants.

A first prototype has been developed, being based on the implementation on a single instrument of a nanoparticle measurement unit able to count particles below 700 nm, an air sampling unit including cascade impactors and filtering units, and a data management software to support the acquisition of data on the concentration of ENMs in real time.

The measurement unit follows the diffusion charging principle, where the airborne particles in the nanometer range captured by the device are electrically charged by corona discharge from a needle-tip electrode set at a voltage high enough to locally ionize the air. The charged particles are subsequently captured on a filter placed inside an electrically isolated Faraday cage where a current meter measures the total current from the charged particles considering that the total current depends on the particle number concentration and the particle size. The software application developed is designed to support the capture of monitoring data from sensors in real-time, real-time QA/QC for data imports, data storage including automatic incremental backup strategies, graphical display, as well as a range of analytical tools for exposure and risk analysis. A scheme of the systems is depicted on figure 1.

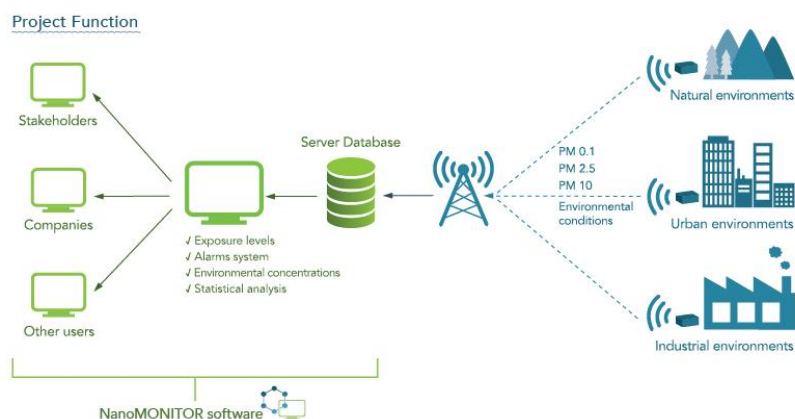


Figure 1. NanoMONITOR systems scheme

By developing a real-time information and monitoring system NanoMonitor supports the risk assessment of nanomaterials under REACH. The authors would like to acknowledge the LIFE program for funding this project (Grant Agreement LIFE14 ENV/ES/000662).



**#1508**

**Effects of azelaic acid in nanovesicles on cell lines selection**

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New drugs development is complicated, costly and time consumptive process, preclinical and clinical trials, apply of new drug and it was approved by FDA. Liposomes and niosomes are nanovesicles which have been widely used as drug delivery. The encapsulation of drugs in these vesicular systems offers several advantages, the modification of the lipophilicity and hydrophilicity, decreasing of toxicity, increasing of stability, circulation time and absorption of the drug. In safety assessment of azelaic acid (AA) in nanovesicles for pharmaceutical was determined. AA was encapsulated in niosomes and liposomes with the compositions of non-ionic surfactant/cholesterol and phosphatidylcholine/cholesterol, respectively. AA and AA in nanovesicles, using MTT assay on three cancer cell lines comparing with vincristine, were investigated. AA incorporated in liposomes more potent than AA, and less potent than vincristine. AA incorporated in nanovesicles was more effective than AA to kill cancer cells, AA- nanovesicular formulations on epidermis cell line in normals, AA encapsulated nanovesicles were moderated when compared with cisplatin. Plain of both nanovesicles showed no growth inhibition. AA incorporated nanovesicles has been proved to has antiproliferative effect in cancer cell lines, the safety of AA when incorporated in nanovesicles has been showed no toxicity to normal cell lines

**Key words:** azelaic acid, nanovesicles, SRB assay, MTT assay

## **INDUSTRIAL INNOVATION LIAISON (i2L) – NSC-SUBGROUP AS SUPPORTING INFRASTRUCTURE FOR REAL-LIFE APPLICATION OF NANOSAFETY ASSESSMENT**

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Nanotechnology represents an enabling technology with a potential for major breakthroughs in diverse industrial sectors. However, processes at the nanoscale, where novel material properties occur, have also been linked to new or different potential risks, that are still not fully understood.<sup>3,4</sup> To ensure the responsible usage of nanomaterials (NMs) along the entire value chain of industrial innovation processes, safety-related issues have to keep pace with emerging technological advances.

The two main bottlenecks that hinder the responsible development of nano-innovations are: 1) knowledge and information on nanosafety is not established consistently and reproducibly across different NMs and biological end points; and 2) innovation and safety are not well enough connected.<sup>5</sup> Thus it is crucial to make a collective and concerted effort to effectively use the multitude of existing nanosafety-relevant data generated throughout European or national programs. Otherwise, safety work will remain explorative, and continue lagging behind innovation.<sup>6</sup>

To create links between safety work and industry, BNN initiated a sub-working group named “Industrial Innovation Liaison (i2L)”<sup>7</sup> which is part of the WG9 “Safe-by-design (SbD) and industrial innovation” of the NanoSafety Cluster. Additionally, this group will support technical development in the European Pilot Production Network (EPPN). The group brings together all nanosafety-relevant experts from pilot-line and innovation-led projects. Hence, real-life and application-oriented nanosafety as well as SbD expertise from WG9 are closely linked and connected. The added value to all participating members is equal access to nanosafety knowhow and innovation-relevant information (e.g. needs, barriers; solutions), thus aiming to create a paradigm shift in bridging innovation and safety. Furthermore, nanosafety experts increase the societal and economic impact of their work by tackling the needs and barriers on the way to the market.

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<sup>3</sup> Aruna, K. et al. "A Systematic Review on Nanomaterials: Properties, Synthesis and Applications." *i-Manager's Journal on Future Engineering and Technology* 11.2 (2015): 25

<sup>4</sup> Nel, A. et al. "Environmental health and safety considerations for nanotechnology." *Accounts of chemical research* 46.3 (2013): 605-606.

<sup>5</sup> Falk, Andreas, et al. "Research roadmap for nanosafety-Part III: Closer to the market (CTTM)." (2016).

<sup>6</sup> Westra J. et al. "Assessing Health and Environmental Risks of nanoparticles – an overview". RIVM report 2014-0157. National Institute for Public Health and the Environment, Bilthoven, The Netherlands (2015)

<sup>7</sup> <http://www.nanosafetycluster.eu/working-groups/industrial-innovation-liaison-i2l-wg10.html>

Within this contribution, the gained knowledge related to risk assessment and safety-oriented product design, a valuable and project-tailored nanosafety concept, will be presented. Based on existing state-of-the-art safety assessment approaches/tools (ECETOC TRA<sup>8</sup>, Stoffenmanager Nano<sup>9</sup> and ISO 12901-2<sup>10</sup>) and following the few established approaches in SbD (e.g. NANoREG<sup>11</sup>), the concept supports the safe use of NMs along industrial processes. The approach is currently applied in European projects INSPIRED, Hi-RESPONSE, R2R-Biofluidics<sup>12</sup> in order to compile a risk profile for NMs (i.e., focus on determining exposure and impact levels).

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<sup>8</sup> [www.ecetoc.org/tools/targeted-risk-assessment-tra/](http://www.ecetoc.org/tools/targeted-risk-assessment-tra/)

<sup>9</sup> <https://nano.stoffenmanager.nl/>

<sup>10</sup> ISO/TS 12901-2:2014. Nanotechnologies — Guidelines for occupational risk management applied to engineered nanomaterials — Part 2 Use of the control banding approach (2014).

<sup>11</sup> <http://www.nanoreg.eu/>

<sup>12</sup> <http://www.nano-inspired.eu/>; <http://hiresponseh2020.eu/>; <http://r2r-biofluidics.com/>

## eNanoMapper applications to support the risk assessment of nanomaterials

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The infrastructure developed within eNanoMapper project aims to support the data management in the area of nano safety research and to enable an integrated approach for the risk assessment of nanomaterials. To achieve these, eNanoMapper developed an ontology, a data infrastructure and modelling tools with applicability in risk assessment of nanomaterials. By merging the expertise of partners in statistical and data mining tools and in predictive toxicology, biology and nanotechnology research, eNanoMapper developed resources and tools for predicting toxicity of nanomaterials and worked towards improving the standards in risk assessment of nanomaterials. The ontology includes common vocabulary terms used in nanosafety research and aims to provide a clear explanation of nanostructures based on information relating to their characterization, relevant experimental paradigms, biological interactions, safety indications and the integration of data from existing nanotoxicology sources. To support a collaborative safety assessment approach an infrastructure for data management was developed, with a database which includes functionalities for data protection, data sharing, data quality assurance, search and interfaces for different needs and usages, comparability and cross-talk with other databases. Further, a collection of descriptors, computational toxicology models and modelling tools were developed, to enable the use and integration of nanosafety data from various sources. The project provides also a rich source of information and documentation (e.g. tutorials, webinars, publications) to support and guide the users. eNanoMapper is supported by European Commission 7<sup>th</sup> Framework Programme for Research and Technological Development Grant (Grant agreement no: 604134).

**#1653**

**IN SILICO TOXICOLOGY TEST SERVICES**

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During the eNanoMapper project in silico toxicologies (IST) webservices were adapted in order to provide REST interfaces that adhere to eNanoMapper standards and specifications and supplemented with interactive SWAGGER documentation. This poster will give an overview of ISTs eNanoMapper API compatible REST services and demonstrate its usage with practical examples

**#1654**

**ENM-ONTOVIEWER: INTERACTIVE VISUALISATION OF SPARQL QUERIES FOR  
ENANOMAPPER ONTOLOGIES AND DATA**

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Within the eNanoMapper project in silico toxicology (IST) maintains the RDF database with nanoparticle ontologies and data. In order to simplify the combined search for data and ontologies in the eNanoMapper RDF backend, we have developed an interface for the visualisation of SPARQL queries. This poster will present the enm-ontoviewer application together with a usage example and links to source code, documentation and docker images.

**#1655**

## **INTEGRATION OF IN SILICO TOXICOLOGY SERVICES IN THE ENANOMAPPER INFRASTRUCTURE**

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During the eNanoMapper project in silico toxicologies (IST) public services were adapted to the specification of the eNanoMapper API and new nanoparticle specific developments were implemented. This poster will depict the interaction of IST services with eNanoMapper resources of other partners which covers use cases like data download/mirroring, augmentation of GUIs with ontology queries and nanoparticle descriptor calculation.

**#1656**

## **IN SILICO TOXICOLOGY SERVICES FOR NANOPARTICLE RISK ASSESSMENT**

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During the eNanoMapper project in silico toxicologies (IST) public server infrastructure was adapted to the requirements of the eNanoMapper project and augmented with new developments. This poster will give a comprehensive overview all IST resources developed within eNanoMapper. Each service will be presented with a brief description and links to the public interface, source code, documentation and download links for self-contained docker images.



#1502

### 12 DESIGN PRINCIPLES FOR 'GREEN NANO

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The intention of many EU projects is the development of new tools for risk and sustainability assessment of nanotechnologies. One of the main goals of the SUN project was the development of an integrated SUN Decision Support System (SUNDS) based on nano-EHS data and methods and intended for practical use by industries and regulators. For SUNDS information and data are needed from toxicological and risk analysis and LCA. It can be used for the assessment of nanotechnology based products and processes in the design and production phases. (see Figure).

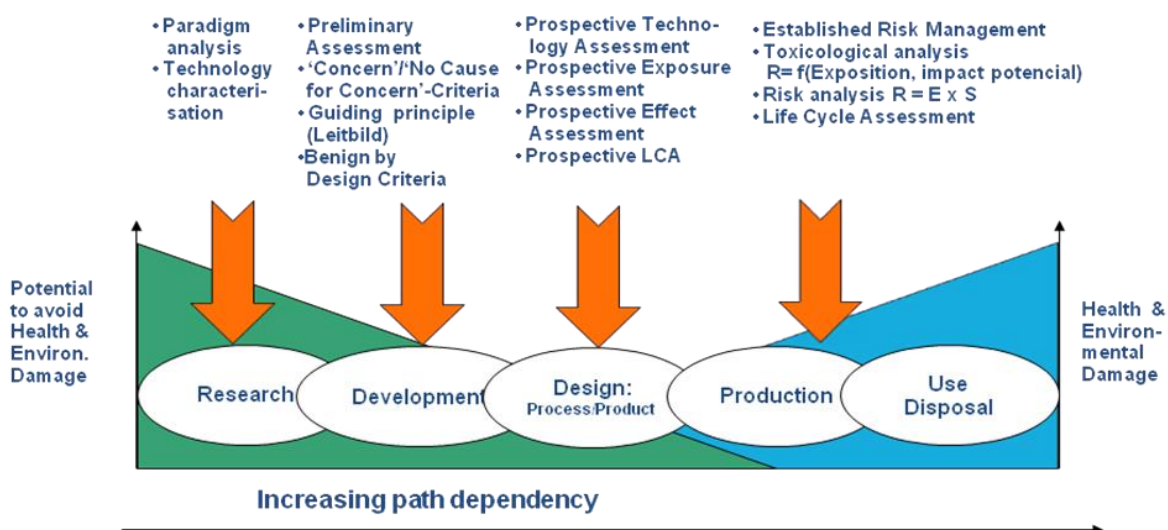


Figure: Approaches to technology assessment and design along different phases of innovation

The development of materials, products and processes based on the next generation of functionalized nanomaterials is still in an early phase of development. In view of the enormous knowledge problems with which prospective technology assessment is confronted, the importance of concurrent approaches to specific developments has to be emphasized. In early phases when applications and their contexts are still unknown, the focus must be laid on what is already known, the technology, the materials and their functionalities and, if already in view, the products and processes that are based on them. In order to realize the precautionary principle, criteria and guiding principles for the precautionary design of green resp. sustainable nanotechnologies, materials, products and processes are needed.

This contribution will give an overview about different 'green' and 'safer' design approaches and will present 12 design principles for 'Green nano' based on the work of the German NanoKommission and it will present additional steps for their operationalization. These design principles are covering four main fields; biomimetics, risk minimization, resource efficiency, and environmentally friendly use of nanomaterials, processes and products in energy and environmental technologies.

## ASSESSING SUSTAINABILITY OF NANO-ENABLED PRODUCTS THROUGH THE LIFE CYCLE

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Clear operationalization and strategy for sustainable nanotechnology is just starting to be addressed (1-4). While development of safe products is pinpointed as a key element in the overall sustainability of nanotechnology (5-7), the inclusion of economic and social aspects of sustainability have also been emphasized by stakeholders (4,8,9). Apart from supporting individual stakeholders in developing more sustainable nano-enabled products, tools for risk management and sustainability tools are also envisioned in playing a role in nanotechnology risk governance (2,10).

In this context, in the frame of the SUN project, a sustainability assessment methodology was developed and implemented in the SUN Decision Support (SUNDS) system as one of the modules in its higher tier, named Socio Economic Assessment (SEA) (2).

The SEA module compares scenarios of nano-enabled products to relevant alternatives with respect to their sustainability aspects (environmental, economic and social costs and benefits) through the lifecycle (2). The SEA methodology aims to pinpoint hotspots (i.e. high risks and impacts or low benefits) that allow the user to see in which ways the sustainability profile of a nano-enabled product can be improved. The unit of analysis is the scenario covering the whole life cycle of a nano-enabled product i.e. synthesis of functional components, product manufacturing, consumer use and product end of life (disposal, recycling and reuse). Within this scenario, the SEA module aims to account for salient sustainability aspects such as transformation of pristine nanomaterial to diverse nano-forms (which constitute different exposure agents), environmental (including human) targets, material and energy fluxes contributing to environmental impacts, economic inputs and social context.

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## CONTROLLING THE HUMAN HEALTH AND ECOLOGICAL RISKS OF NANO-ENABLED PRODUCTS THROUGH THE LIFE CYCLE

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While the global nanotechnology value chain is expected to reach \$4.4 trillion by 2018 (1), and nanosafety research funding has been on a rise since 2005, large uncertainties about the environmental, health and safety (EHS) risks of nanomaterials (NM), including physicochemical characterization, environmental release, exposure and hazard estimation and risk characterisation, continue to persist (2).

In order to address these emerging issues, European Chemicals Agency (ECHA) has adopted a bottom-up approach in REACH implementation through two working groups (i.e. NanoMaterials Working Group and Group Assessing Already Registered Nanomaterials). The registration of the nano-form of chemicals has been mandated to be performed separately from the bulk form, and REACH guidance documents are being updated for NM (3). While ECHA's efforts and other activities may eventually lead to effective guidelines to control NM risks, there is a need to implement state-of-the-art risk control through the nano-enabled product lifecycle.

There are two key challenges to be addressed in implementing risk control through the nano-enabled product lifecycle. The first concerns the application of appropriate Technological Alternatives and Risk Management Measures (TARMM) to address the risk posed by a specific nano-form in an exposure context. The second concerns implementation of risk control in a cost effective manner, as even explicitly recognized by regulations (e.g. REACH Authorisation's Analysis of Alternatives and Socioeconomic Analysis) and policy prescriptions (e.g. European Commission's Precautionary Principle).

In this context, in the frame of the SUN project, a risk control methodology that addresses the issues highlighted above was developed and implemented in the SUN Decision Support (SUNDS) system as one of the modules, named Risk Control (RC), in its higher tier (4). The RC module supports the control of human health and ecological risks by assessing risk control strategies (so called TARMM) through nano-enabled product lifecycle (4,5).

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## USING KNIME AND PYTHON TO ACCESS AND PROCESS ENANOMAPPER DATA

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The constantly increasing production of engineered nanomaterials (ENMs) has constituted the use of data collection and management, as well as non-testing methods largely important. eNanoMapper (<http://enanomapper.net/>) provides a computational infrastructure for both management and modelling of ENM toxicological data using RESTful web services. However, the need has arisen for local usage of this data. To this end, in this work, we describe how to access eNanoMapper data using KNIME and Python, since KNIME allows reproducible and transferable workflows. More specifically, it is shown how to create a well-structured cURL command for eNanoMapper data services, the conversion to Python, how to formulate Python requests and handle responses. Finally these scripts are embedded in a KNIME workflow which allows additional manipulation options such as formatting and visualization. The aim is to create a community contribution repository where users may upload their workflows and scripts, thus allowing all eNanoMapper functionalities to be made available locally via easy-to-use pipelines.

## BUILDING A WEB-BASED RISK ASSESSMENT AND RISK MANAGEMENT TOOL – NANOMATERIALS TREATMENT TECHNOLOGIES

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Nowadays, nanomaterials are widely used in cosmetics, food, pharmaceuticals or electronics industries. These little particles, that have a diameter less than 100 nm, can be added to the product in order to improve the product developing new applications. However, the increasing exposure to the NPs increase the risk for both human health and the environment. Studies have shown that NPs can produce adverse effects generating reactive species capable of producing oxidative stress that can lead to cell death. Conventional treatment technologies can be applied to remove NPs from waste streams in order to reduce the exposure. However, these techniques were developed to treat particles with higher diameters and it is unknown exactly which is the NPs removal efficiency due to the few studies addressed in full-scale installations.

GUIDEnano is a European project funded to develop a risk assessment web-based tool. Tool will support end-users during risk assessment and in the definition of effective risk mitigation plans for the effective application of the safety interventions. To reach these goals, the project is building upon the state-of-the-art on risk assessment and management, generating new predictive models, and novel risk management solutions.

The aim of this review is to summarize NPs removal efficiency for the conventional techniques from scientific publications in order to complete the web-based tool, not only taking into account the treatment technique used but also the type of nanomaterial to be treated. With the feedback from the industrial partners of the project, a practical case in which a list of their activities for production processes and waste streams with NPs were detailed. For each one, a detailed list of the characteristics of this waste, environment compartment release, route of exposure, treatment techniques available and their removal efficiency associated were developed. An example of this results for Nano ZnO production and milling and caning as phase can be seen in the following table.

Waste	Phase	Activity	NPs	State	Form	Condition	Technological compartment	Units	Route of exposure	Treatment Technique	Removal efficiency
Milling	Air extraction	Dust	ZnO	Solid	Suspension	Volume	Indoor air	m <sup>3</sup> /h kg/m <sup>3</sup> *h	Inhalation	Scrubber	>99%
										Electrostatic precipitator	99,2 - 99,7 %
		Filters		Solid	Matrix	Surface	Landfill	Kg/day	Dermal	Landfill	100%



			d	Incineration WIP							>99,9%
	Emptying /cleaning	Powder	ZnO	Solid	Matrix	Volume	Landfill	Kg/day	Dermal	Landfill	100%
		PPEs		Solid	Powder	Surface	Landfill	Kg/day	Dermal	Incineration WIP	>99,9%
Canning	Quality control	Can of paint with defects	ZnO	Liquid	Dispersion	Surface	Landfill	kg/day	Dermal	Landfill	100%
										Incineration WIP	>99,9%
		Paint residues	MWCN	Solid	Powder	Surface	Landfill	kg/day	Dermal	Landfill	100%
										Incineration WIP	>99,9%
			TiO2	Liquid	Dispersion	Volume	Landfill / Waste water treatment plant	m3/day mg/m3*day	Dermal / oral	Activated sludge	>90%
										Anaerobic digestion	High
										Microfiltration and ultrafiltration	>99%
										Water treatment plant	1-52%

## DEVELOPMENT OF A RISK ASSESSMENT STRATEGY FOR THE GUIDENANO TOOL

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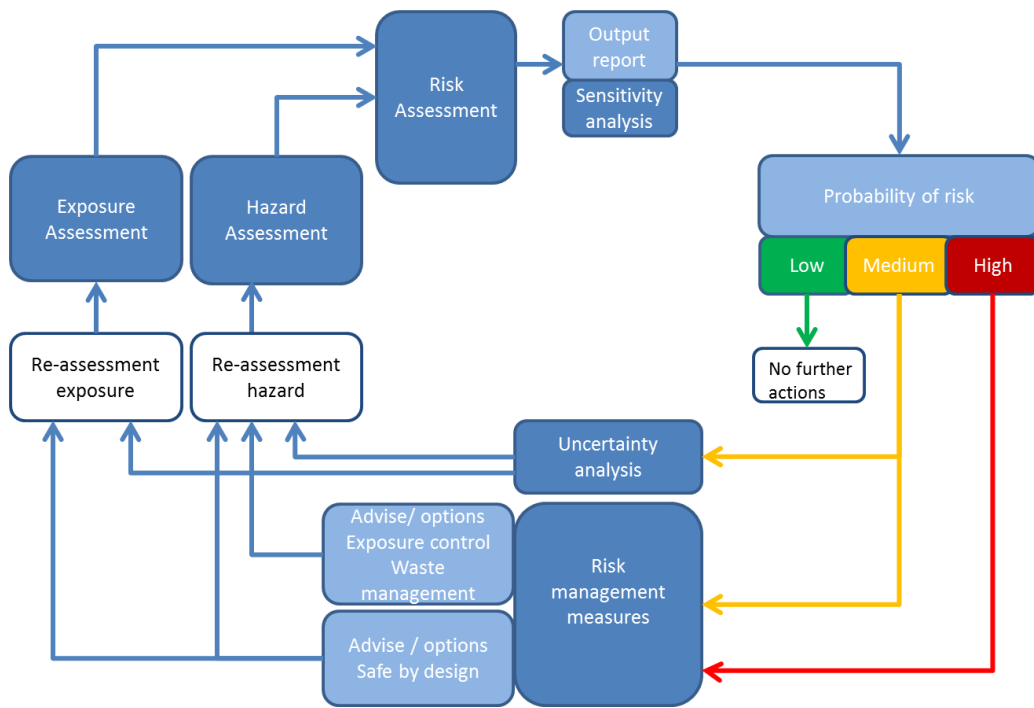
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One of the main goals of work package 7 of the GUIDEnano project is to develop a risk assessment strategy for an NM-enabled product during its development and before introduction on the market. This risk assessment strategy is incorporated in the interactive web-based GUIDEnano Tool, which will guide the NM-enabled product developers (mainly industry) into the design and application of the most appropriate risk assessment and mitigation strategy for a specific product. The strategy is currently built into the GUIDEnano tool and has been evaluated with hypothetical and real case studies within the project.

For development of the strategy to assess the risk of NMs, information on existing risk assessment methodologies was used, together with discussions with experts from inside and outside the project. The strategy can be divided in four main elements (see Figure 1):

1. Input and information requirements (hazard and exposure assessment)
2. Risk assessment (calculation of a risk ratio and classification into three risk categories)
3. Follow-up actions (reduction of uncertainty, risk mitigation)
4. Output report.

A sensitivity analysis of the entire risk assessment process has been developed to identify the key assumptions or uncertainties to be reduced throughout this process. Furthermore, we are currently working on the contents of the output report of the GUIDEnano tool as well as on the first outlines of an evaluation of the tool by industry and other stakeholders.



## A WEB-TOOL BASED APPROACH TO EVALUATE THE QUALITY OF AN (ECO)/TOXICITY STUDY PERFORMED WITH NANOMATERIALS

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The final goal of the GUIDEnano project is to develop a web-based guidance tool, which will help users to design and apply the most appropriate risk assessment and mitigation strategy for a specific nanomaterial (NM). The project is developing innovative methodologies to evaluate and manage human and environmental health risks of nano-enabled products, considering the whole product life cycle. A hazard assessment strategy has been proposed that relies on existing information, using data from studies that are reliable and adequate based on well-defined criteria for quality, relevance and similarity of the NM used in the study compared to the exposure relevant material.

The approach proposed to assess the quality of an (eco)/toxicity study performed with NMs builds upon the proposal of Card and Magnuson (2010), which combines a 'study score' and a 'nanomaterial score'. However, this proposal focuses only on human toxicity studies, does not include clear criteria for the NM characterization data to be provided, and how to categorize the scores. For the GUIDEnano tool, the proposal was modified as follows:

1) The approach was made applicable for both human and ecotoxicity studies by introducing questions on organism characterization, study design description and results documentation, taking the ToxRTool as a basis. The questions were different for eco and human toxicity studies and for *in vitro* and *in vivo* studies. The answers to these yes-or-no questions result in a score of the reliability (K) of the study.

2) The data that should be provided on the NM and their dynamics throughout the study was established, i.e. their identification (i.e. source, chemical composition), concentration, purity, stability, protocols of dispersion, exposure medium characteristics and a minimum set of physicochemical properties of the pristine NM and of the NM in the exposure medium. Based on the reporting of this data a substance (S) score is derived.

3) The combination of the K and S scores results in a final quality (Q) score for the study which will categorize the study as unacceptable, medium, high or very high quality.

The proposed quality score approach has been tested and improved by evaluating several scientific articles on different endpoints of human toxicity (mutagenicity, carcinogenicity and inhalation toxicity) and ecotoxicity (aquatic and sediment compartments), covering a broad range of different NMs and years of publication and taking into account the number of representative articles available per endpoint. The results obtained helped to identify and refine the weak points of the quality tool and to improve its structure to be sufficiently restrictive.

**Acknowledgements** - FP7 project GUIDEnano (agreement n° 604387).

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**REVIEW OF HUMAN RISK ASSESSMENT MODELS FOR MANUFACTURED NANOMATERIALS APPLICABLE DURING DIFFERENT STAGE GATES OF PRODUCT INNOVATION. FIRST RESULTS FROM EU H2020 ‘CALIBRATE’ PROJECT/WP2**

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The EU H2020 caLIBRAte project (<http://www.nanocalibrate.eu/>) aims to design, calibrate and implement a next generation systems-of-systems risk governance framework for manufactured nanomaterials (NM). Aim of this framework is to allow for screening of existing and emerging risks, quantitative risk assessment for humans and the environment and implementation of safe-by-design decision support systems and risk management. A central part of the project is to support the assessment of NM along the “Cooper Stage-Gate®” product innovation model. This idea-to-launch innovation model allows for the screening and scoping of ideas, proceeding towards product development, testing, launch and post launch review. It is also applicable to NM, either during the development of NMs themselves, or for development of end-products involving the application of NM. An important part of this framework is concerned with human risk assessment models (WP2), in which integrated hazard, exposure and risk assessment models (HRA models) are being adopted and further developed for stage-gate specific requirements.

Here, the initial results are presented. First, in consultation with external stakeholders from SMEs, industry, government, stage-gate specific criteria have been defined to which (existing) HRA models need to comply. These involve general criteria, such as transparency of the model, operational costs, duration, capacity to deal with data gaps, compatibility of the model to accept new data etc.. More specific criteria for HRA models

were also defined concerning both the model output (e.g. type of risk indicators, type of uncertainty characterization provided, accuracy of resulting risk estimate) and model input criteria (e.g. various physical chemical characteristics, the inclusion of kinetic endpoints, in vitro or in vivo hazard endpoint included, exposure duration, aggregated exposures, inclusion of non-intentional use). Secondly, human hazard (e.g. as further developed in Nanosolutions), exposure (a.o. Consexpo, Consexpo Nano, dART, FIOH Indoor Air Quality Model) and control banding, risk assessment models and decision support tools (a.o. Stoffenmanager Nano, NanoSafer, Guidenano, SUN) are now being evaluated for their applicability towards the different stage gates of innovation bearing these predefined criteria in mind. Steps towards further refinement of the existing HRA models for application along the stage gate model as well as potential inclusion of novel more predictive approaches (e.g. physicochemical modelling, internal dose assessment and PBPK modelling systems toxicology, high throughput screening, adverse outcome pathway based approaches, bioinformatics) will be discussed.

## **NEW TOOLS TO SUPPORT THE MITIGATION AND CONTROL OF THE EXPOSURE TO ENMs IN THE WORK PLACE**

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Within the LIFE NanoRISK project, two new tools have been developed to assist SMEs, large companies and professional users on the selection of proper technical measures and personal protective equipment when dealing with ENMs. These tool comprise: 1) a computerized RMM library, and 2) a multimedia guideline on recommended measures to control the exposure to ENMs in the workplace.

This Risk Management Library is offered as a tool to support the selection of adequate and experience based personal protective equipment (PPE) and engineering controls (EC) for preventing exposure to nanomaterials and release in the workplace. The current version of the RMM library is available as a downloadable Microsoft Excel® file, providing the user with data on the effectiveness of common Engineering Controls, Administrative Controls and Personal Protective Equipment when dealing with ENMs under a wide range of operations and processes, ranging from lab scale activities to industrial processes. The tool includes operation buttons, data inputs modules, and calculation operators, all of them integrated to allow stakeholders to estimate the efficiency of existing measures and/or get recommendations on how to reduce the exposure under specific operative conditions and ENMs.

The guidance on recommended measures developed particularly assists companies in the selection of adequate and experience based personal protective equipment (PPE) and engineering controls (EC) to preventing exposure to nanomaterials and release in the workplace. The guidance includes interactive figures, downloadable videos and links specifically designed to support the achievement of the main objective of the guide, the selection of adequate measures to control the exposure to ENMs and prevent release into the environment.

The following sections are included in the current version:

1. Abbreviations and acronyms
2. Summary
3. Introduction: Environmental, health and safety (EH&S) issues in Nanotechnology.
4. Regulations and standards
5. Basics on Risk Management Measures: STOP principle
6. Effectiveness of common risk management measures against occupational exposure to ENMs

7. Recommended measures for the safe handling and control of exposure
8. Health Surveillance and environmental monitoring
9. Instruction sheets
10. Frequently asked questions
11. Annexes

The authors would like to acknowledge the LIFE program for funding this project ( Grant Agreement LIFE12 ENV/ES/000178)



**#1635**

## **NanoDESK – Advanced web based tools to promote the application of nanotechnology and safe use of nanomaterials in the plastic industry**

Sofia Ricarte, Carlos Fito-López, Rafael Gozalbes, Riccardo Concu, Robert Rallo, Esteban Santamaria

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Within the NanoDESK project, a new web based platform to promote the use of ENMs in the plastic industry will be developed. This platform includes: 1) an observatory on the safety of nanostructured polymer based materials, 2) a suite of 4 computational models (QSAR/ QNTR models) to predict the toxicity of ENMs, 3) a new integrated tool to evaluate the exposure in the workplace, relevant environmental compartments and consumers, 4) an Advanced Data mining / information searching tool, and 5) a strategic plan and road map of the plastic sector.

NanoDESK focuses on the promotion of the nanotechnology as Key Enabling Technology to support the development of new added value plastic materials based on the use of engineered nanomaterials (ENMs). To this end, a comprehensive suite of user-friendly tools to reduce the potential impact of engineering nanomaterials (ENMs) on the environment and human health will be developed.

The outcome will be a web-based software platform for facilitate sustainable manufacturing of ENMs, providing tools to support the identification and development of low-toxicity ENMs, the evaluation of the consumer worker and environmental exposure on a regulatory basis, and the selection of strategies to reduce and control the impact of ENMs on human health and the environment.

The platform will consist of 3 subsystems: optimized computational models for the hazard assessment of ENMs; exposure estimation models to quantify the exposure following REACH provisions, and a decision support system to guide the industry on the safe use.

The new models to be developed will be based on the use of new and/or refined mathematical algorithms to describe cause-effect relationships between physicochemical properties and toxicity, likelihood of release of ENMs (i.e. emission rates) during relevant production process involving ENMs, as well as rate constants to model common reactions such as dissolution, sedimentation, homoaggregation, heteroaggregation, and/or degradation in the workplace and the environment.

The use data mining algorithms (classification, imputation, dimensionality reduction-feature extraction and pattern recognition) will be key to support the identification of reliable and robust data on the hazards and exposure potential to ENMs. These data will be generated using ISA TAB format, supporting the further transference to existing databases.

The authors would like to acknowledge Interreg SUDOE program for funding this project ( Grant Agreement SOE1/P1/E0215 )

## **Recommended technical, organizational and personal protective equipment to control the exposure to ENMs in research laboratories**

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Knowledge, data, and test methods on PPE and technical measures for NMs are still very limited, even though the first commercial products specifically advertised for protection against NM have started being marketed.

Most of the research activities related with the evaluation of the effectiveness of risk management measures have been limited to respiratory protective equipment, and only a limited number of studies have reported information on the effectiveness of technical measures, chemical protective gloves and/or protective clothes. In this study, conducted under the framework of the GUIDEnano project, the efficiency of laboratory fume hoods and protective dermal equipment against metal oxide based nanomaterials in dry form and dispersed into a liquid matrix was evaluated.

A vertical flow laboratory fume hood and a suite of different protective gloves against chemical and biological risks, with distinct materials and thickness, were tested. The types of protective gloves selected were: latex (without powder), vinyl (with and without powder), nitrile (two thickness), and reusable, such as neoprene, polyvinyl chloride or butyl.

The evaluation of the performance of the abovementioned measures was conducted under controlled conditions, including temperature, pressure and humidity. The evaluation of the effectiveness of chemical protective gloves against nanomaterials was based on the procedures defined on ISO/CD 19918 "Protection against chemicals-Measurement of cumulative permeation of chemicals with low vapour pressure through materials". The evaluation of the effectiveness of fume hoods was conducted following the procedures defined on the ES harmonised standard EN 14175-4:2005 "Fume cupboard. Part 4. On site test method" and ASHRAE 52 2007 "Method of testing general ventilation air-cleaning devices for removal efficiency by particle size.

The results shows that the performance depends strongly on the material of the glove, and although generally there are no pores in their surface, some small defects or gaps can be enough to offer a way in to the glove.

The data retrieved from the analysis of the performance of the fume hood showed an average efficiency of  $99.04 \pm 0.36$  %. The experimental studies conducted demonstrated that a proper hood design together with

an adequate airflow are key to ensure the capture of much of the nanoparticles released to the work-place environment.

The authors would like to acknowledge the EU Commission FP7 program for funding this project (Grant agreement No: 604387).

## NanoMILE QSARs, grouping and mechanistic hazard assessment platform

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A brief overview of the WP9 results within NanoMILE will be presented. This includes the computational evaluation of nanoparticles' (NPs) properties and their correlation with biological/physiological experiments to develop robust mechanistic Quantitative Nanoparticle – Activity Relationship (QNAR) models for NP hazard and risk assessment. In particular, extensive quantum mechanical (QM) calculations and microscopy image analyses for a series of metal and metal oxide NPs yielded a comprehensive set of descriptors related to the morphology (size, shape, colour) and the energetics (band gap, HOMO/LUMO, electronegativity, etc.) of the NPs. These results were combined with relevant biological evaluations to construct reliable QNAR models for hazard predictions of metal/metal oxide NPs. Furthermore, predictive QNAR models were developed with the application of in-house tools (Enalos KNIME nodes) for more complex systems (i.e., NPs with surface or protein corona modifications) by collecting and integrating data from the literature. Importantly, to facilitate the use of our tools for NP risk assessment within the broad research community, each QNAR model has been exported as a freely-accessible web service through the Enalos InSilicoTox Platform ([insilicotox.com](http://insilicotox.com)).

A second major outcome from NanoMILE WP9 is the development of rules and guidelines for safety-by-design (SbD) of nanomaterials, linked to the broad experimental data as well as to the QNAR models described above. An overview of key guidelines is presented as well as the NanoMILE computational tools to support SbD approaches for industry and regulators.

## **HUMAN HEALTH RISK ASSESSMENT MODULE IMPLEMENTED IN SUNDS**

### **RISK ASSESSMENT & MANAGEMENT**

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The SUNDS (SUN Decision Support system) web application software has been developed in the SUN European project on Sustainable Nanotechnologies ([www.sun-fp7.eu](http://www.sun-fp7.eu)). The software aims at supporting decisions on assessment & management of nanomaterials and nano-enabled products in industry, regulatory bodies and insurance companies. Within SUNDS, the Human Health Risk Assessment module implements a quantitative HHRA methodology for the estimation of deterministic and probabilistic risks caused by nanomaterials along different life cycle stages (synthesis, formulation, use and end of life).

In the SUNDS HHRA module, risk is evaluated by combining the outputs of the effects (hazard information) and exposure models. The outputs of these models, as well as the resulting risk, can be estimated deterministically or probabilistically, depending on data availability.

Risk is estimated for a combination of aspects including a lifecycle stage, a specific target, a specific activity (where applicable) and a defined route of exposure. This combination has been called "lowest unit of assessment" (LUA). With the aim of providing integrated information on the estimated risks for specific life cycle stages or for the entire lifecycle of a specific nanomaterial, aggregations methodologies are required. The aggregation methodologies implemented in the HHRA module produce a single risk value for each lifecycle stage as well as for the entire lifecycle considering all lifecycle stages, targets, activities and routes of exposure (i.e. all assessed LUAs). Moreover, they may be additive (in the case of risks related to the same target) or non-additive (in the case of risks related to different targets for the same lifecycle stage).

The HHRA methodology has been applied to different case studies including nano-copper oxide -based biocidal paint, plastic car bumper coloured with nano-organic pigment, nano-Silver used in antibacterial polymer fibres in textiles, Titanium Dioxide ( $\text{TiO}_2$ ) for self-cleaning coating for ceramic tiles. The focus of this contribution will be on the results of the application of the developed HHRA methodology to the case study of nano-copper oxide -based biocidal paint.

#1526

### THE NANODEFINER E-TOOL FOR NANOMATERIAL CLASSIFICATION

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The NanoDefine project addresses the issues on the availability of suitable measurement techniques, reference materials and validated methods for nanomaterials, following the European Commission's definition recommendation (European Union, 2011).

NanoDefine's objectives include the development of the NanoDefiner e-tool, a web-based decision support framework developed to assist laboratory workers as well as experts in nanomaterial classification and labelling/registration processes. Centralized as well as intranet installation is possible. Its goal is to recommend suitable measurement techniques for a specified material as well as to enable (non)-nano decisions based on the obtained results.

To be capable of this, a robust, extensible and easily manageable knowledge base, maintainable by non-computer scientists, was developed with the help of experts from industry and academia. Knowledge about measurement technique criteria and particulate component criteria is stored in well-defined and standardised attributes, matched by rule sets via the Drools Rule Engine. Importance was placed on the capability of handling uncertainties and ensuring transparency in decision making.

A guided workflow leads through the whole process:

i. Dossier description: A dossier is created, stating the purpose and describing the mono- or multiconstituent sample.



ii. Particulate component definition: Knowledge base attributes are used to define particulate components. A changed criterion in the user interface triggers the decision making, providing reasoned live feedback on recommended measurement techniques.

iii. Method conduction: Recommended methods can be conducted ad libitum. Afterwards, analysis results can be imported on which a (non-)nano decision is based.

iv. Report generation: Detailed information on the sample, its particulate components and results of conducted methods are aggregated in a purpose-oriented report for labelling/registration organisations.

The prototype of the NanoDefiner has reached a closed beta status and is currently being evaluated by NanoDefine internal users with positive results. An open beta phase with evaluation by external partners will start in February 2017.

A poster/presentation will cover the workflow, knowledge base, decision making and reasoning. Additionally, a hands-on demo of the NanoDefiner's workflow will be shown.

#### References:

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## NOVEL HIGH RESOLUTION DIFFERENTIAL MOBILITY ANALYSER (HRDMA) FOR SIZE CHARACTERIZATION OF NANOPARTICLES SMALLER THAN 5NM

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Nanomaterial characterization is a challenging subject due to limitations in analysis method and also in sampling. Aerosol technology has been detecting and characterizing nanoparticles for long time and some of their developments can be of use in nanotechnology.

Having accepted and validated methods to determine several characteristics and descriptors of NanoParticles (NPs) is an open challenge, being size one of them. Since the nanoparticle range, as the European Commission's definition recommends (Recommendation 2011/696/EU), stands between 1 and 100 nm, very different properties are found in this huge range of sizes, therefore different methods will be required for the extreme ranges. It is accepted that NP size affects tremendously its properties, toxicity among them. There is an added difficulty since no on line standard method is accepted for the size determination of the smallest range in nanoparticles, despite Electron Microscopy (EM) methods are the most accepted ones.

Cylindrical Differential Mobility Analysers (DMAs) are used for determining the size distribution of bigger NPs. However, for the smallest ranges, technical challenges are found due to the high mobility of the smallest charged NPs that drives to higher losses and low sensitivity. Additionally, the resolution (ability to distinguish NPs of similar sizes) of the cylindrical DMAs has limits with the smallest NP sizes. To solve the technological challenges of the DMAs for the smallest NPs, RAMEM has developed a parallel plate DMA in which the NPs follows shorter and simpler trajectories, resulting in higher transmission, minimum losses and high sensitivity. The instrumental design has allowed a resolution 4 times higher than the classical cylindrical DMAs.

The inversion between mobility and size is needed because with the smallest NPs, the charging stage is an open challenge. With the smallest NPs the charging efficiency is very low and some of the charged particles have multiple charges. Having a high yield of charged NP with only one charge avoiding radioactive charger and neutralizers is a challenge for this size range. There are neutralizers in the market but their performance for the smallest NPs is still open. RAMEM is investigating different alternatives in order to achieve a well know equilibrium charge distribution in the smallest nanoparticles to univocally link the mobility with the size.

RAMEM will present results of DMA characterization: Resolution and Transmission for the smaller NPs range using mobility. Some neutralization experiments results will also be shown for the NPs in the smaller range. The successful completion of the neutralizer will allow the size classification of the smaller NPs, launching an instrument that fills the gap in size characterization for NPs smaller than 7nm. The resulting instrument will be able to distinguish NPs with differences of 2% for the smallest NPs.

## QCM-BASED METHOD TO PREDICT IN VIVO BEHAVIOUR OF NANOPARTICLES

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In biological fluids, proteins and other biomolecules bind to the surface of nanoparticles to form a coating known as the protein corona which in turn becomes primary determinant of the nanoparticles' fate and behavior. Here we develop a QCM-based platform and methodology to obtain data from interactions of nanoparticles with selected human plasma proteins. Polystyrene and gold particles coated with transferrin are immobilized on QCM sensor chips and by means of a 'sandwich' format binding assay, functional protein epitopes capable for biological recognition on the surface of the particles can be quantified as measured by the increase of the sensor's resonant frequency. The link between physical - chemical properties (size and surface charge) of nanoparticles and availability of such recognition fragments under dynamic flow conditions is investigated. The methodology allows monitoring of the interactions at the nanoparticle surface in real-time and assessment of the kinetics of the interactions and the affinity with different biomolecules. Our approach may be applied to a large range of nanoparticles and biomolecules providing a set of important info on the behavior of nanoparticle complexes in biological systems.

## Atomic Ordering Determined by Choice of Iron Salt Precursor in Continuous Hydrothermally Prepared Fe-Co Bimetallic Nanoparticles

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Fe-Co oxide nanoparticles were prepared by a continuous hydrothermal method with iron nitrate and ammonium iron citrate as alternative iron precursors. The degree of crystallinity, the Fe/Co ratio, and the elemental distribution inside the synthesised nanoparticles were found, by XRD, STEM-HAADF imaging, -EDX analysis, and -EELS mapping, to be very different when the iron salt was changed. The EDX results demonstrated that the Fe/Co ratios detected in the particles synthesised with iron nitrate were close to the ratios in the metal salt solution. However, the Fe/Co ratios of nanoparticles synthesised with ammonium iron citrate were found to be always higher than that in the solutions. STEM-EELS mapping revealed that the distribution of cobalt and iron in the iron nitrate synthesised nanoparticles was inhomogeneous (Figure 1a) and that the nanoparticles synthesised with ammonium iron citrate were uniform (Figure 1b). This indicates that the presence of ammonium citrate could inhibit the precipitation of Co ions in solution, and promotes the crystallisation of Fe-Co bimetallic nanoparticles, leading to a homogeneous composition distribution. The elemental distribution of iron and cobalt in the particles may have an effect on not only the magnetic properties but also their biocompatibility.

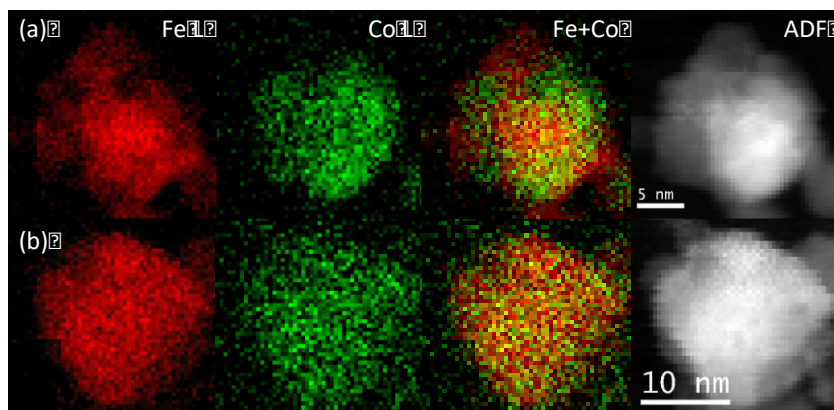


Figure 1 STEM-EELS maps for Fe-Co oxide nanoparticles (a) synthesised with iron nitrate, and (b) ammonium iron citrate precursors. From left to the right are the Fe and Co L edge maps, the combined Fe and Co maps, and the corresponding ADF images.



**EUROPEAN STANDARDIZATION PROJECT ON DETECTION AND IDENTIFICATION  
OF NANO-OBJECTS IN COMPLEX MATRICES: EVALUATING OF SAMPLE  
PREPARATION METHODS AND CHARACTERIZATION METHODS**

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CEN/TC 352 “Nanotechnologies” Working group 3 “Health, safety and environmental aspects” has launched a preliminary work item namely; Technical Specification: Nanotechnologies – Guidance on detection and identification of nano-objects in complex matrices in 2014. The scope of this Technical Specification is to provide guidelines for detection and identification of specific nano-objects in complex matrices like food, liquid environmental compartments and waste water assuming an a priori knowledge of their nature of the nano-objects like their chemical composition and size distribution.

This project is currently going into its final stage and is one of the first technical standards guiding the user through the whole sample preparation process towards specimen preparation including evaluations for each single sample/specimen preparation step. This Technical Specification covers mainly a set of appropriate methods like Field-Flow-Fractionation, Electron Microscopy, Single-Particle-ICP-MS and alternative characterization methods and describes the combination of them using a similar sample preparation.

Starting with examples for detection and identification tasks in complex matrices an overview of measurement techniques are given, implementing guidance for the selecting a suitable method for a given measurement problem. Measuring principle, performance, uncertainties, sample preparation and interpretation of results are discussed.

The selected detection and identification methods are based on a combination of size classification and chemical composition analysis. Corresponding requirements for sampling and sample preparation are given (matrix destruction, physical separation and detachment processes) where identification can also be supported e.g. by additional morphology characterization. This work provides links to measurement standards if available to aid the user towards a properly use of other nano-characterization methods.

## PREPARATION AND CHARACTERIZATION OF FUNCTIONALIZED MULTIWALLED CARBON NANOTUBES

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This study is part of Nanosolutions Project that ultimately aimed to provide a prototype engineered nanomaterial for safety classification through identification of the characteristic properties that determine their biological hazard potential at the cellular, tissue and organism level. Our hypothesis behind this work is that the interaction of engineered multiwalled carbon nanotubes (MWCNT) with living organism is a complex process that depends not only on the nature of the core MWCNT, but most importantly on the physico-chemical surface properties. For this purpose, a set of stable and well-characterized MWCNT have been synthesized namely; MWCNT-Core, MWCNT-Carboxylate, MWCNT-Ammonium and MWCNT-PEG in order to represent MWCNT with bare, negative, positive and neutral/hydrophilic surface, respectively.

MWCNT were produced and thoroughly characterised by physico-chemical methods of analysis. All basic (TEM imaging, size distribution, surface properties and degree of functionalization) and more advanced metrics were determined. Additionally, a dispersibility protocol and quality assurance system was developed in order to ensure long term stability of the MWCNT dispersion for the end users. In general, all types of MWCNTs showed mostly individually dispersed MWCNT with the possibility of having few aggregates and bundles mainly with MWCNT-Carboxylate and MWCNT-Ammonium. All different types of MWCNT tested formed stable dispersions in 0.1% Albumin at 2mg/ml that enables further biological testing in vitro and in vivo. The stability of MWCNT dispersion was confirmed by macroscopic examination, UV spectroscopy and TEM imaging. Size distributions of MWCNTs (length and diameter) were determined from TEM images and measured approximately an average of 10-20 nm in diameter and 1.0 to 2.0  $\mu$ m in length. Raman spectroscopy indicated that MWCNTs preserve their structure after dispersion. XPS survey scan was performed to study the surface functionalisation of MWCNT and these results were further confirmed with thermogravimetric analysis (TGA). Long term dispersibility studies also confirmed that MWCNT dispersions maintained good stability over time with no significant changes in the parameters tested. In conclusion, we were able to provide a set of stable and fully characterized MWCNT that have been tested for biological hazard potential through Nanosolutions Project WP4-10.

### Hazard assessment along the life cycle of nano-enabled products

**#1501**

#### **NANOPARTICLE SHAPE-DEPENDENT NLRP3 INFLAMMASOME ACTIVATION**

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The widespread and increasing use of engineered nanomaterials (ENM) increases the risk of human exposure, generating concern that ENM may provoke adverse health effects. In this respect, their physicochemical characteristics are critical. The immune system may respond to ENM, amongst others, by inflammatory reactions. Inflammasome activation has drawn significant attention since inflammasomes (especially NLRP3) respond to a wide range of stimuli including NPs, and their activation is associated with various inflammatory diseases, including lung fibrosis, obesity and type 2 diabetes. Inflammasomes are intracellular multiprotein complexes that assemble upon stimulation, resulting in activation of caspase-1 that in turn induces production of interleukin (IL)-1 $\beta$  and IL-18, which are potent mediators of inflammation.

A single Ag ENM as well as Au ENM of different shapes, some of them PEGylated, were extensively characterized, and tested for possible LPS contamination. ENM were pre-incubated with 50% human serum (HS) and tested in the presence of 10% HS. PMA-activated THP-1 cells were used; cell viability and IL-1 $\beta$  production were measured.

Only PEGylated Au nanorods, showed inflammasome activation; seen as a dose-dependent increase in IL-1 $\beta$  and concomitant reduced viability. THP-1 cells deficient in NLRP3 or ASC did not show effects on viability and did not show IL-1 $\beta$  production.

Our data suggest shape-dependent inflammasome activation. These data are in line with Niikura et al. (ACS Nano 2013; 7: 3926) who showed inflammasome activation by Au nanorods but not -spheres or -cubes.



Supported by the EU funded project FutureNanoNeeds (Grant agreement N° 604602).

## The impact of nano-scale and micro-scale zero-valent iron particles on planktonic microorganisms in reservoir water

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Nano-scale and micro-scale zero-valent iron (nZVI and mZVI) materials have shown high potential for the remediation of polluted soil aquifers and groundwaters. However, these particles have been reported to be toxic to bacteria and other microorganisms in cell cultures. The objective of this study was to assess the impact of 100 mg/L of nZVI and mZVI on the planktonic microorganisms from Harcov reservoir. Concretely, bacterial biomass was determined using 16S rRNA gene and the number of cultivable cells and the dominant species of phytoplankton along with cell counting were determined after 0, 1, 3, 7, 14 and 21 days. In addition, the bacterial community structure was also investigated by Next-Generation Sequencing (NGS). Changes in the reservoir water chemistry were also monitored. nZVI particles caused a rapid decrease in oxidative reductive potential (from +200 to – 136 mV) and dissolved oxygen concentration (from 9.2 to 2.3 mg/L) after 1 day. Both gradually increased after 3 days and stabilized during the experiment. Interestingly, the number of cultivable bacteria increased significantly after 3 days and even more after 7 days ( $P < 0.0001$ ), while the total bacterial abundance was comparable to the control. The NGS results are still being analysed. The algae were slightly affected by nZVI only at day 7 ( $P = 0.04$ ), while the cyanobacteria were stable over 21 days. Surprisingly, mZVI did not have any effect on bacteria or algae, but the number of cyanobacteria doubled after 7 days. We also observed that nZVI was oxidized after 3 days and mZVI after 7 days. Bacteria and cyanobacteria might profit from the bioavailable oxidized iron and increase their abundance.

## BIOLOGICAL EFFECT OF PEROVSKITE NANOMATERIAL ON PSEUDOMONAS PUTIDA AND MICROORGANISMS IN ACTIVATED SLUDGE

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Perovskite nanomaterials have become very popular in development of solar cell technology over the last few years due to their high potential in the efficiency improvement and cheap manufacturing. Organometal lead halide perovskites thus have prospect to be widely used in photovoltaics. Theoretically, the perovskite material could be released into the environment during the solar cell production and handling. Therefore, we performed several experiments to assess the biological effect of this material on soil bacteria *Pseudomonas putida* and mixed culture of microorganisms in activated sludge from waste water treatment plant. Two types of perovskites were tested -  $\text{CH}_3\text{NH}_3\text{PbI}_3$  and  $\text{CH}_3\text{NH}_3(0.33)\text{CH}_3\text{NH}_3(0.67)\text{PbI}_3$  both containing toxic lead which can leak from the material. The concentration of the lead was thus determined using inductively coupled plasma mass spectrometry. Perovskite nanoparticles were characterized using scanning electron microscopy, differential centrifugal sedimentation and Zetasizer. Biological effect of this material on metabolism of microorganisms and cell viability was assessed using respirometry, bacterial growth rate and fluorescence analysis. Four concentrations of perovskites were tested on *P. putida* – 5, 10, 50 and 100 mg/L and three higher concentrations were used to measure respiration of microorganisms from activated sludge – 50, 100 and 500 mg/L. The lead in form of  $\text{Pb}(\text{NO}_3)_2$  was used as a control to distinguish if concentration of leaked lead was toxic for microorganisms or not. The lead from  $\text{Pb}(\text{NO}_3)_2$  was added in four concentrations 100, 200, 500 and 1000 mg/L to the bacterial culture of *P. putida* to determine growth rate. Diluted Soya nutrient broth was used as a medium for *P. putida*. Microorganisms of activated sludge were tested in medium which was prepared according to the standard methodology ČSN EN ISO 9408 with the concentration of dry matter of activated sludge 30 mg/L. Perovskite nanomaterials did not cause any toxic effect against *P. putida* and microorganisms in activated sludge. Bacterial growth rate was negatively affected only by 500 and 1000 mg/L of lead from  $\text{Pb}(\text{NO}_3)_2$ , both concentrations being much higher than the actual concentrations of leaked Pb from perovskites. The highest tested concentration of lead without any toxic effect was 200 mg/L. Overall, the two perovskite nanomaterials was found to be safe when in contact with tested microorganisms.

## HAZARD ASSESSMENT OF AEROSOLIZED GRAPHENE-RELATED MATERIALS IN A 3D HUMAN ALVEOLAR TISSUE *IN VITRO*

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Graphene and related materials (GRM) have promising combination of properties which allow for a wide range of new high-technology applications such as in electronics, photonics, and energy storage<sup>1</sup>. Thus, the mass production of GRM has taken up in the past several years and their products are already arriving on the market-place. Hence, it is imperative to assess their possible interaction with humans during the life-cycle and the potential hazards of GRM to human health under realistic exposure scenarios<sup>2</sup>. Concerns have been raised especially regarding their interaction with the respiratory system, as being the primary route of exposure for airborne particles<sup>3</sup>. It has been shown that GRM are easily respirable and that they can avoid the clearance mechanisms of the respiratory system, meaning that following inhalation, GRM can deposit beyond the ciliated airways which can result in the induction of oxidative stress and pulmonary inflammation<sup>4</sup>. In the present study a 3D human lung model composed of epithelial cells, dendritic cells and macrophages<sup>5</sup> combined with an aerosolization system has been used for a simulation of realistic exposures of inhaled GRM to the lung epithelial barrier tissue, and evaluation of oxidative stress, cell viability, and pro-inflammatory response. The effect has been assessed upon exposure to GRM at different concentrations, corresponding to relevant GRM doses after inhalation exposure<sup>6</sup> (ranging from 0.2 to 1.5 µg/cm<sup>2</sup>). GRM aerosolization has been performed using the commercially available nebulizer VitroCell®Cloud system, coupled to the Quartz Crystal Microbalance for assessment of the aerosolized material deposition. First outcomes have shown that nebulization of graphene oxide (GO) resulted in a dose-dependent material deposition, and none of the investigated parameters were elevated for the tested concentrations in the 3D lung model. Further investigations are ongoing to assess effects after a prolonged exposure to GO and compare to that of different types of GRM, such as graphene nanoplatelets, which are forecast to represent a majority of the market value of the graphene market in the year 2026<sup>7</sup>.

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**FROM EXPOSURE TO IMPACT:  
NANOECOTOXICITY STUDIES IN THE NEMATODE *C. ELEGANS***

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Through exponentially growing production and application of engineered nanomaterials (MNMs) it is becoming clear that nanoparticles are released into environment where they interact with a biota in a nanoparticle specific manner. Toxicity is one of the outcomes of these interactions. We use the soil nematode *Caenorhabditis (C.) elegans* in order to identify biological and environmental impacts of MNMs. *C. elegans* is abundant in the soil ecosystem and one of the key invertebrate animal models in biomedical research. Thus, it serves as an evident tool for nanoecotoxicity studies. The transparency of worms facilitates uptake routes and bioavailability of labelled MNMs by imaging. We show that the distribution of MNMs in respective organs/ tissues correlates with their function. Silica nanoparticles induce premature aging phenotypes on the behavioural level, as a reduction of pharyngeal pumping and an increase of internal hatch, and on the molecular level as a widespread protein aggregation.

Protein aggregates range from induction of amyloid in nucleoli of intestinal cells to facilitation of protein aggregation in body wall muscles and axons of neural cells. Within the European Consortium NanoMILE we screen pristine and modified MNMs such as Ag, ZnO and CeO<sub>2</sub> in different species in order to identify common pathways across a wide range of wildlife taxa (algae, *C. elegans*, arthropods, fish, rodents). We show that liquid cultivation in 96-well microtiter plates promote a clumping behaviour that is characteristic for wild worms. Thus, the designed microhabitat in 96-well plates mimics the worm's natural habitat and serves as an optimal screening platform. By comparing multiple endpoints in *C. elegans* such as life span and age-resolved behaviour as well as neurotoxicity studies we show that Ag MNMs induce significant dose-dependent toxicity. Adverse effects of CeO<sub>2</sub> or ZnO MNMs were not observed in our chronic exposure studies.

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**ENDOTHELIAL RESPONSES OF THE ALVEOLAR BARRIER *IN VITRO* IN A  
REALISTIC DOSE-CONTROLLED EXPOSURE TO DIESEL EXHAUST  
PARTICULATE MATTER**

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Exposure to fine and ultra-fine environmental particles is still a problem of concern in many industrialized parts of the world and the intensified use of nanotechnology may further increase exposure to small particles.

The response triggered by air pollutants is not limited to local effects of the respiratory system but is often systemic, resulting in endothelial dysfunction or atherosclerotic malady. The link between air pollution and cardiovascular disease is now accepted by the scientific community, but the underlying mechanisms responsible for the pro-atherogenic potential still need to be unravelled in detail.

A complex tetraculture system was developed (Figure 1) and consists of the alveolar type-II cell line (A549), differentiated macrophage-like cells (THP-1), mast cells (HMC-1) and endothelial cells (EA.hy 926), seeded in a 3D-orientation on a microporous membrane to mimic the cell response of the alveolar surface *in vitro* in conjunction with native aerosol exposure (VitrocellTM chamber).

After exposure the expression of different anti-oxidant target genes and inflammatory genes such as HMOX1, NQO1, ICAM1 or VCAM1 as well as the nuclear translocation of Nrf2 was evaluated.

In addition, the potential of diesel particles to induce the upregulation of CYP1A1 mRNA in the endothelium was analysed.

The endothelial cells responded to the treatment also with the upregulation of CYP1A1 mRNA and nuclear translocation of AhR.

Overall, exposure triggered a response in the endothelial cells after indirect exposure of the tetraculture system to low doses of particles, underlining the sensitivity of ALI exposure systems.

The use of the tetraculture together with the native aerosol exposure equipment may finally lead to a more realistic judgment regarding the hazard of new compounds and/or new nano-scaled materials in the future.

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## Effect of coating on the *in vivo* toxicological potential of CeO<sub>2</sub> NPs

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Ceria nanoparticles (CeO<sub>2</sub> NPs) have several industrial applications and pharmacological potential due to their antioxidant properties. However, toxicity data on CeO<sub>2</sub> NPs are scarce and show contradictory results. In the present study uncoated, polyethylene glycol- and citrate-coated CeO<sub>2</sub> NPs (4-8 nm; PlasmaChem) were administrated to C57Bl/6 mice by repeated dose (3x) pharyngeal aspiration using four different doses of each NPs (corresponding to 4.4, 8.8, 17.6 and 35.2 µg Ce<sup>2+</sup>/mouse/aspiration), and sampled 1 and 28 days after the last administration. DNA damage was assessed by the comet assay locally in bronchoalveolar lavage (BAL) and lung cells, and systemically in liver cells. Micronuclei, a biomarker of chromosome damage, were analysed in bone marrow and peripheral blood erythrocytes. Immunotoxicity was evaluated by BAL cell counting. Furthermore, histopathological effects on the lungs and biodistribution of the NPs (analysis of Ce<sup>2+</sup> in several organs) were assessed. At 24-h, a significant increase in DNA damage was induced at the highest doses by uncoated and citrate-coated NPs in BAL cells but not in lung cells. Significant but not dose-dependent effects were observed in lung and liver cells by these NPs at 28-d. PEG-coated NPs induced not significant DNA damage in any cell type at any exposure time. No systemic genotoxic effects in bone marrow or blood leukocytes were observed with either of the NPs or exposure times. A dose-dependent accumulation of macrophages and activated lymphocytes was seen in the lungs for all the NPs, although a milder reaction was elicited by the coated NPs. Our findings show that short-term exposure of mice to CeO<sub>2</sub> NPs induces pulmonary inflammation and a non dose-dependent DNA damage but not chromosome damage, (Funded by the EU FP-7 GUIDEnano, Grant Agreement No. 604387).

## Prediction of the mutagenic potential of nanomaterials within the GUIDEnano hazard assessment

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During the last few years, several theoretical models have been developed for assessing the potential risks of engineered nanomaterials. The final goal has usually been to identify safe exposure levels to which workers and consumers could be exposed without health consequences. This kind of quantitative approach is appropriate for most of the human toxicological endpoints considered in current regulations (e.g. REACH) but not applicable to endpoints without an identified effect-threshold - such as mutagenicity. The outcome of qualitative risk assessment is not a numerical limit but a statement (e.g. positive, negative or equivocal). Here, we propose a theoretical approach to assess the mutagenic potential of nanomaterial-enabled products throughout their lifecycle, which could be incorporated into a web-based risk assessment tool. Following a 3-step hazard assessment strategy developed within the EU FP7 project GUIDEnano, we focus on describing the criteria for evaluating and weighting existing literature information based on current knowledge on the relevance and limitations of assays used in the genotoxicity and mutagenicity testing of nanomaterials. The similarity between the exposure relevant material and the tested one, and the quality of the study are checked using common algorithms developed in GUIDEnano for all types of (eco)toxicological endpoints. A third parameter, the relevance of the assay used, is specific for the mutagenicity endpoint. Different in vivo/in vitro studies and types of genotoxic effects are weighed to provide a final statement and to describe its associated uncertainty. We try to make use of all information available, including studies that do not comply with conventional test guidelines. Such a framework might help in developing more efficient screening strategies that avoid exhaustive testing of new nanomaterials (Funded by the EU FP7 GUIDEnano, Grant Agreement No.604387).

**Nanoparticles, ions and shape governing soil microbial functional diversity:  
nano shapes micro**

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Nanoparticles (NPs) are known to affect microbial metabolic processes at single cell level or lab-culture strains. However, the impact of different NPs properties such as the particle, ion release and shape on functional responses of natural soil microbial communities remain poorly understood. We assessed the relative importance of particles and ions in bacterial toxicity and how the functional diversity of soil microbial communities were impacted by shapes (i.e. plates, spheres and rods) in laboratory incubations. Our results showed that the relative contribution of NPs(particle) increased with increasing exposure concentrations (accounting for about 60-68% of the total toxicity at the highest exposure level). In addition, the functional composition of the microbial community differed significantly according to different NP shapes. The various properties of NPs thus can significantly and differentially affect the functional composition of microbial communities and associated ecosystem processes depending on the level of environmental exposure. Experiments were performed with Ag, Cu, and ZnO NPs as well as with Pb-based perovskites. These particles represent an important part of the NPs commonly used nowadays and display a broad range of different physico-chemical properties on top of morphological similarities.

## COLLOIDAL CHARACTERIZATION OF NANO-SIZED PIGMENTS IN (ECO)TOXICOLOGICAL MEDIA

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Engineered nanomaterials are embedded in different matrices, such as plastics, coatings and cosmetics. Nano-sized pigments are added to different consumer polymer-based products, such as polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET) etc., to give them color.<sup>1</sup> The nano-particulate structure enhances color fastness and persistence in application with respect to bulk material.<sup>2</sup> However, the risk assessment of nanopigments is still a challenge, calling for an additional physico-chemical characterization. In this context, within the EU FP7 SUN project, the colloidal stability of organic nano-sized pigment PRED 254 and Fe<sub>2</sub>O<sub>3</sub> pigment was investigated in biological and environmental media, relevant for (eco)toxicological testing. In particular, centrifugal separation analysis (CSA), combined with dynamic and electrophoretic light scattering (DLS and ELS) and Transient Resistive Pulse Sensing (TRPS) techniques, have been employed to assess the long-term stability of the tested nano-dispersions. The colloidal characterization performed in the tested media may allow to correlate physicochemical properties of nano-sized pigments with principal factors determining the (eco)toxicological outcomes and estimate their behavior in real environments. The release of sub-micro fractions from nanocomposite materials (nanopigments embedded in a polymeric matrix) has also been investigated to estimate the possible exposure scenarios occurring at different life cycle stages.

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Acknowledgement - The authors gratefully acknowledge support from the European Union Seventh Framework Programme [FP7/2007-2013] under ECGA No. 604305 "SUN".



## Toxicological Response Of Healthy And Asthmatic 3D in vitro airway model To CuO Particles At The Air-liquid Interface

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In this study we investigated the biological effects of three concentrations of CuO and carboxylated CuO nanoparticles on human 3D in vitro airway model of healthy and asthmatic origin.

The human 3D in vitro airway model consist of human stem cells and fully differentiated human ciliated respiratory and goblet cells. The epithelial lining is covered by a mucus layer that is produced by goblet cells and moved by ciliary beating. The cells form a layer with an intact integrity (at least in the healthy inserts), and are apically exposed to air, allowing a realistic exposure to a test atmosphere.

CuO was tested at low, mid, and high concentrations of 23, 120, 470 mg/m<sup>3</sup> for CuO and of 32, 128, 495 mg/m<sup>3</sup> for CuO-COOH respectively. Results were obtained on the exposure characteristics (SEM, deposition, APS/SMPS). Exposure of cells from healthy (n=3) and asthmatic origin (n=5) resulted in visual observable particle deposition. Cells of asthmatic origin showed a less homogenous cell layer compared to the cells of healthy origin.

Aerosol exposures to CuO showed 5% cytotoxicity (LDH) at the highest concentration in the cells of healthy origin. The cytotoxicity response of the cells of asthmatic origin shows a dose response which reaches around 15% toxicity at the highest concentration. Cytokines (IL-8, IL-6 and MCP-1) showed a dose response in both cells of healthy and asthmatic origin, after exposure to CuO. In the highest concentration applied the response of the healthy cells is higher, which might be due to more cells (due to less cytotoxicity) compared to the asthmatic cells.

Aerosol exposures to the carboxylated CuO (CuO-COOH) resulted in 5% cytotoxicity (LDH) at the highest concentration in the cells of healthy origin. The cytotoxicity response of the cells of asthmatic origin shows a dose response wich reaches around 10% toxicity at the highest concentration. Cytokines (IL-8, IL-6 and MCP-1) showed a dose response in both cells of healthy and asthmatic origin, after exposure to CuO-COOH. In the highest concentration applied, the response of the healthy cells is lower for CuO-COOH compared to CuO exposure.

We conclude that human 3D in vitro airway models of healthy and asthmatic origin can be used to study the biological effects of different forms of CuO nanoparticles.



## IN VITRO STUDIES TO INVESTIGATE THE RELATIONSHIP BETWEEN DIFFERENT COATINGS AND THEIR EFFECT ON NANOPARTICLE TOXICITY

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Surface coatings of nanoparticles (NPs) are usually applied to selectively change a NP's specific properties, e.g. dissolution, aggregation or to endow the NP with functionalities for specific applications. The presence and type of coating on the surface of NPs may affect their uptake, translocation and toxic potential. Within the GUIDEnano project, we aim to establish relationships between different types of coatings and their effects on NP toxicity.

To fulfil this aim, a set of two core materials TiO<sub>2</sub> and CeO<sub>2</sub> each with three different coatings (citrate, polyethylene glycol (PEG) and dodecylphosphonic acid (DDPA)) were tested in a battery of *in vitro* tests. To evaluate cytotoxicity (membrane integrity and metabolic activity), inflammation (generation of reactive oxygen species, secretion of TNF- $\alpha$ ) and cellular uptake (TEM analysis), THP-1 macrophages were exposed to the different NPs for 24h. To evaluate genotoxicity (comet assay at non-cytotoxic concentrations) 16HBE human lung epithelial cells were exposed to the different NPs for 24h. Translocation over the intestinal barrier was studied in a Caco-2 cell model with the set of CeO<sub>2</sub> NPs only, using a 48 h exposure time. For all assays the nominal concentrations of the NPs were based on the NP core and were in the range 0.3  $\mu$ g/ml to 100  $\mu$ g/ml.

In both macrophages and lung epithelial cells neither the uncoated nor the coated CeO<sub>2</sub> and TiO<sub>2</sub> NPs induced cytotoxicity in the presence of serum in the cell culture medium. Besides, no significant secretion of TNF- $\alpha$  was detected for neither of the studied NPs. TEM images revealed that both CeO<sub>2</sub> and TiO<sub>2</sub> (with and without coatings) existed mainly as aggregates/agglomerates and were distributed mainly in endocytic vesicles and lysosomes. The comet assay revealed that the NPs did not induce genotoxicity in the 16HBE lung epithelial cells. However, when serum was omitted from the cell culture medium, all particles induced a certain level of cytotoxicity in both THP-1 macrophages and 16HBE cells. Different coatings appeared to play a limited protective role in inducing cytotoxicity. In addition, tail moments in the comet assay were increased for all particles, at concentrations below those inducing cytotoxicity. Here, citrate coated materials were more genotoxic than uncoated and PEG coated materials. No generation of ROS was detected for any of the NPs below cytotoxic concentrations. In the Caco-2 cell barrier model, no translocation of Ce<sup>2+</sup> was observed for any NP after 48h of exposure. However, some factors that could lead to an underestimation of the *in vitro*



absorption were identified: i) the large agglomerate particle size in cell culture media for uncoated NP and the hydrophobic properties of DDPA coated NPs, ii) the Caco-2 monolayer system may not cover all mechanisms of intestinal NP translocation, namely lack of transcytosis across M-cells.

In summary, there was no clear, uniform trend in coating-dependent cellular uptake, translocation over the gut barrier, cytotoxicity, inflammation and genotoxicity for CeO<sub>2</sub> and TiO<sub>2</sub> NPs in the *in vitro* studies performed. Interestingly, the presence or absence of serum played a more significant role in determining the effects than the type of coating, complicating interpretation of the results and underscoring the need for standardization of the most appropriate experimental set up, i.e. the one most representative of real-life exposure for each exposure route.

(Funded by Grant Agreement No.604387).

## ENANOMAPPER TUTORIALS: GUIDANCE TO THE INFRASTRUCTURE FOR NANOMATERIAL MODELING

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The aim of the eNanoMapper FP7 project is to design infrastructure for nanosafety based on open source tools. In that spirit, the code and tools are available to all, together with detailed tutorials that have been produced in order to provide users with instructions on their operation.

The applications that have been developed within eNanoMapper are complemented by an array of support materials, publicly available on the eNanoMapper website, produced to suit the needs of users (experimentalists, data managers, etc.) and developers.

The eNM series of tutorials are mainly represented by electronic documents (e.g. in pdf format) or video materials. The information included in the tutorials is also a result of the developer-user interactions and represent a guideline based also on the experience accumulated during the development, testing and deployment phases. However, apart from these documents, each application has specific guidance and support materials available (additional resources, help sections, etc.). The collection of tutorials developed within the project is accessible at: <http://www.enanomapper.net/enm-tutorials>.

The tutorials are developed alongside with the applications, therefore they may also be updated to follow new releases of software. A repository has been set at: <https://github.com/enanomapper/tutorials>, to enable

access to the tutorials' files in various formats and facilitate long term and open access to different versions of the tutorials after the end of the project and over further developments. All eNanoMapper tutorials are distributed under the license CC-BY 4.0.

Additionally, a list of terms used within the project was prepared and made publicly available, with the same aim to help the users in understanding and implementing specific elements related to ontology, data management, modelling and risk assessment (available at: <http://www.enanomapper.net/library/enm-dictionary>).

**Effect of coating on the *in vivo* toxicological potential of CeO<sub>2</sub> NPs**

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Ceria nanoparticles (CeO<sub>2</sub> NPs) have several industrial applications and pharmacological potential due to their antioxidant properties. However, toxicity data on CeO<sub>2</sub> NPs are scarce and show contradictory results. In the present study uncoated, polyethylene glycol- and citrate-coated CeO<sub>2</sub> NPs (4-8 nm; PlasmaChem) were administrated to C57Bl/6 mice by repeated dose (3x) pharyngeal aspiration using four different doses of each NPs (corresponding to 4.4, 8.8, 17.6 and 35.2 µg Ce<sup>2+</sup>/mouse/aspiration), and sampled 1 and 28 days after the last administration. DNA damage was assessed by the comet assay locally in bronchoalveolar lavage (BAL) and lung cells, and systemically in liver cells. Micronuclei, a biomarker of chromosome damage, were analysed in bone marrow and peripheral blood erythrocytes. Immunotoxicity was evaluated by BAL cell counting. Furthermore, histopathological effects on the lungs and biodistribution of the NPs (analysis of Ce<sup>2+</sup> in several organs) were assessed. At 24-h, a significant increase in DNA damage was induced at the highest doses by uncoated and citrate-coated NPs in BAL cells but not in lung cells. Significant but not dose-dependent effects were observed in lung and liver cells by these NPs at 28-d. PEG-coated NPs induced not significant DNA damage in any cell type at any exposure time. No systemic genotoxic effects in bone marrow or blood leukocytes were observed with either of the NPs or exposure times. A dose-dependent accumulation of macrophages and activated lymphocytes was seen in the lungs for all the NPs, although a milder reaction was elicited by the coated NPs. Our findings show that short-term exposure of mice to CeO<sub>2</sub> NPs induces pulmonary inflammation and a non dose-dependent DNA damage but not chromosome damage, (Funded by the EU FP-7 GUIDEnano, Grant Agreement No. 604387).

## FREE RADICAL GENERATION AND ATHEROSCLEROTIC EFFECTS OF REDOX MODIFIED COBALT OXIDE NANOPARTICLES

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Given the prominent cardiorespiratory effects of nanoparticles in air pollution, the potential for manufactured nanomaterials (MNMs) to have cardiovascular actions requires urgent attention. Generation of oxidative stress is likely to play a crucial role in the biological mechanisms of nanoparticles. Here, we investigate the cardiovascular actions of redox-modified cobalt oxide nanoparticles, hypothesising that these nanoparticles will promote the vascular disease atherosclerosis *in vivo*, the extent of which will be related to their redox potential and ability to generate superoxide free radicals.

Cobalt oxide (Co<sub>3</sub>O<sub>4</sub>) nanoparticles were doped iron, by incorporation of Fe<sub>2</sub>O<sub>3</sub> into the crystal structure. Free radical generation from MNMs (acellular) was determined using electron paramagnetic resonance following reaction with the superoxide-selective spin-trap, Tempone-H. 100%Co MNMs generated superoxide free radicals in distilled water at high concentrations (>10 µg/mL). 100%Fe MNMs produced significantly greater levels of superoxide, with Fe-doped-Co<sub>3</sub>O<sub>4</sub> producing intermediate levels.

Three forms of Co/Fe (100%Co, 100%Fe & 1.5Co:1.5Fe) were investigated in Apolipoprotein-E knockout (ApoE<sup>-/-</sup>) mice, that generate atherosclerotic plaques in their major arteries. Mice were fed a 21% fat Western diet for 8 weeks to accelerate the development of plaques. In the final 4 weeks of feeding, mice were repeatedly instilled with MNMs (35 µg twice weekly) or the vehicle by aspiration. Exposures are underway at the time of abstract submission. Mice will be sacrificed ~16 h after the final instillation, taking blood and lavaging lungs to measure cell differentials and inflammatory cytokines. The thoracic aorta will be stained en-face with SudanIV to quantify the intimal coverage of lipid-rich plaques. The brachiocephalic artery will be used for in-depth histological assessment (size, structure and composition) of atherosclerosis. Plaques in this region exhibit a 'complex' phenotype that provides indications plaque instability that would underlie a coronary event in man. Biopsies of lung, liver, spleen, kidney, lymph nodes will be taken for additional pathological analysis.

We believe that the upcoming results of these investigations will provide useful insight into whether MNMs can promote the vascular disease atherosclerosis, and whether redox potential of particles is a key indicator of biological action. This is the first time the vascular effects of MNMs with different redox potential have been investigated using nanoparticles from a single parent material, and will contribute valuable data towards employing a safe-by-design approach to the design of new MNMs.

*This work was funded by a Seventh Framework Programme (FP7) from the European Commission, ("NanoMILE", NMP4-LA-2013-310451). Studies were approved by local research ethics committee and in accordance with the Animals Scientific Procedures Act (UK Home Office).*

## INFLUENCE OF SIZE AND SURFACE FUNCTIONALISATION ON THE GENOTOXICITY OF GOLD NANOPARTICLES.

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Gold nanoparticles (Au NPs) have drawn attention due to their particular properties at the nanoscale, which have increased the interest in this nanomaterial for applications in e.g. controlled drug delivery, cancer treatment, biomedical imaging, or diagnosis (Cabuzu et al., 2015).. Although elemental gold is considered to be inert, several studies have shown that Au NPs can be genotoxic both *in vitro* and *in vivo* (Sabella et al., 2011; Geffroy et al., 2012). However, Au NPs currently produced present a wide range of sizes and functionalisations, which could affect their interactions with the environment or with biological structures and modify their toxic effects. In this study, we investigated the role of size and surface charge in determining the genotoxic potential of Au NPs, by analyzing their ability to induce DNA and chromosomal damage in human bronchial epithelial BEAS-2B cells. Two core sizes (3.5 nm and 20 nm) and three functionalisations representing different surface charges – carboxyl for negative charge, amino for positive charge, and PEGylated (polyethyleneglycol coated) for neutral charge – were used in this study.

DNA damage was assessed by the alkaline comet assay and chromosomal damage by the cytokinesis block micronucleus assay, as previously described (Catalán et al., 2016). 50.000 and 250.000 BEAS-2B cells were seeded in 24-well and 6-well plates (for the comet assay and the micronucleus assay, respectively) 48 h prior to the start of the treatment. Four different doses, chosen on the basis of preliminary cytotoxicity tests, were prepared for each type of Au NPs using dispersions sonicated (in a bath) for 5 min. Treatment time was 24 h for the comet assay and 48 h for the micronucleus assay (cytochalasin B added 6 h after the beginning of the treatment).

In the 3.5-nm core Au NPs, the amino-functionalised form was clearly genotoxic in both assays, while the PEGylated Au NPs produced a weak DNA damage induction only at the highest dose tested. Carboxylated 3.5-nm core Au NPs did not have genotoxic effects. In the case of the 20-nm core size, the amino-functionalised Au NPs induced DNA damage but not micronuclei. The PEGylated and carboxylated 20-nm Au NPs did not produce DNA damage, but the PEGylated form increased the frequency of micronuclei at all doses tested. The carboxylated 20-nm Au NPs gave a statistically significant increase in micronuclei only at the lowest dose tested.

According to our results we can conclude that particle size and functionalisation modulate the genotoxicity of Au NPs in BEAS-2B cells *in vitro*.

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## The effect of physicochemical properties on the toxicity and intracellular uptake of Gold Nanoparticle on exposed BEAS-2B cells

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Numerous potential biomedical applications of gold nanoparticles (AuNPs) have made it necessary to study their toxicity. Moreover, the introduction of ligands carrying different functional groups on these AuNPs has introduced changes to their surfaces to allow for additional range of biomedical applications, which will at the same time influence their cellular recognition and toxicity. The present study has therefore investigated the toxicity and intracellular uptake of approximately 3 and 15 nm AuNPs functionalized with either –methyl ammonium (-NH<sub>2</sub>), -carboxylate (-COOH) or polyethylene glycol (-PEG).

Human bronchial epithelial cells (BEAS-2B) were exposed to AuNPs with different functional groups and their toxicity was assessed using xCELLigence RTCA impedance based technology and intracellular uptake was investigated using the CytoViva<sup>®</sup> dark-field hyperspectral imaging system.

The results obtained indicated that both the 3 and 13 nm AuNPs functionalised with -NH<sub>2</sub> adversely affected the viability of the cells. The 3 nm -COOH and -PEG functionalised AuNPs produced relatively no toxicity, while the 15 nm -COOH and -PEG functionalised AuNPs produced moderate toxicity. The 3 and 15 nm AuNPs functionalised with -NH<sub>2</sub> were taken up more readily than those functionalised with -COOH and -PEG. Intracellular uptake for both the 3 and 15 nm -COOH functionalised AuNPs was observed, while little or no uptake was observed for both sizes of -PEG functionalised AuNPs.

**Conclusions:** It can be concluded that surface functionalization and size of NMs may influence their toxicity and their intracellular uptake.

**An integral study of graphene material safety on the eukaryotic model  
*Saccharomyces cerevisiae*.**

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Graphene and the large ensemble of related structures (graphene family nanomaterials, GFN) have gained a great interest during the last years in different fields -i.e. engineering or biomedicine - due to its unique electronic, mechanical, thermal and optical properties<sup>1</sup>.

Given their increasing applications, there is a growing likelihood of GFN release into the environment, which could lead to a human and environmental exposure with potentially harmful effects. Nevertheless, although GFN are nowadays commercialized, there is still an important lack of consensus in the results of the published studies evaluating their toxicity. Moreover, the available literature focuses on the effect of limited types of GFN, and only in acute or short term exposures<sup>2</sup>, while the long term effects remain unknown. All considered, it is not possible to come to a definitive conclusion regarding GFN safety.

Our work aims to fill the gap in systematic and comparative studies concerning different types of GFN. Here we are carrying out the first *in vitro* study, comprehensive and comparative, to evaluate the toxicity of the most relevant GFN types, currently available in the market, using the unicellular eukaryotic organism *Saccharomyces cerevisiae*. This fungus is one of the most widely used eukaryotic models to understand basic molecular processes in humans and other higher eukaryotes, and is increasingly used in the toxicity assessment of chemicals such as heavy metals and engineered nanomaterials<sup>3, 4, 5</sup>.

This study evaluates, at short and medium-long term, cell viability, cytotoxicity, genotoxicity, in cells exposed to GFNs. In the short-term we are evaluating the preliminary toxicity through growth-curves, while the acute cytotoxicity is assessed using the LDH assay and FUN-1 staining tests. In the medium-long term analysis we are evaluating the genotoxicity through the comet assay. Herein we present the first results of our work.

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## **Acknowledgments**

The project leading to this application has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 691095 and from Junta de Castilla y Leon, project No BU079U16.

## The potential impact of engineered nanomaterials (ENMs) on marine ecosystems: investigations using the marine mussel *Mytilus sp*

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The occurrence, potential hazards, and particular effects of ENMs in marine environments are not sufficiently understood. Research focusing on the potential impacts of ENM contamination in the receiving marine environment lags behind those conducted in freshwater environments. The marine bivalves, such as the blue mussel *Mytilus sp.*, act as filters of the sea, pumping large volumes of water and processing various particulates including, if present, ENMs. Therefore, they may act as important indicators of ENM contamination. A sub-chronic (21-day) study was performed to represent a low-level, medium-term exposure to ENMs (CuO and TiO<sub>2</sub>) in the marine environment. The key questions addressed were: 1) whether mussels accumulate ENMs during sub-chronic exposure and if so, in which body tissues, 2) whether ENM accumulation can be related to adverse health effects (monitored via oxidative stress parameters, genotoxicity and haemocyte lysosomal membrane stability), 3) if mussels are able to eliminate ENMs from their body and at what rate, and 4) whether ENM functionalisation influences accumulation/excretion kinetics and/or toxicity. Environmentally relevant concentrations of CuO and TiO<sub>2</sub> ENMs (0.3 mg/L and 20 µg/L, respectively) were used within exposures. Both CuO and TiO<sub>2</sub> ENMs included those with no functionalisation (core), and those functionalised with polyethylene glycol, carboxylic acid and ammonia. Results indicate that the gill is the main site for CuO ENM accumulation (3 times the amount of Cu in CuO COOH ENM exposed mussels in comparison to controls), while the digestive gland is the main site of TiO<sub>2</sub> ENM accumulation (13 times the amount of TiO<sub>2</sub> in TiO<sub>2</sub> core ENMs than controls). These findings indicate that bivalve, filter-feeding species, such as *Mytilus sp* are significant targets for NP exposure and are extremely important when considering their toxicological impact in the aquatic environment.

The authors would like to acknowledge all collaborators and project partners within the European FP7 funded NanoSolutions project, which has funded this work under grant agreement no 309329.

## **Impact of silver and silver nanoparticles on the structure and functionality of microbial communities in sewage treatment plants**

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Silver nanoparticles (AgNPs) are amongst the most commonly used nanoparticles in consumer products. Therefore they will inevitably end up in the environment, mostly through the entry pathways sewage sludge to soils (application to fields as a fertilizer) or effluent to freshwater systems. However, due to the antimicrobial properties of silver, the microbial communities in the sewage treatment plants might be affected and therefore the refunctional efficiency of the treatment processes. Standard tests to evaluate the effects of environmental contaminants on microbial (sludge) communities (e.g. OECD 209) consider only a short term exposure (average contact time 3 hours). As silver is non-biodegradable it has the potential to accumulate in the environment and have long-term effects on microbial communities. Consequently, the aim of this study was to compare the effects of two concentrations (1 mg/L and 5 mg/L) of ionic silver (tested as silver nitrate, AgNO<sub>3</sub>) and one concentration (5 mg/L) of different AgNPs (coatings and size) as well as Ag<sub>2</sub>S NPs (to mimic environmentally relevant form of AgNPs) on the community structure over a longer time frame of 4 weeks. Three commercially available silver nanoparticles were selected (Amepox, Poland): a) uncoated and paraffin stabilized with a primary particle size of 3-8 nm b) PVP coated ; size 50 – 60 nm c) Ag<sub>2</sub>S NP (approx.. 50 nm ; PVP stabilized). The aqueous AgNP stock suspensions were characterized with DLS, NTA, TEM (size distribution and homogeneity) and AAS (for dissolved and total silver concentration). The diversity of the bacterial community and potential structural changes were analyzed with 16S rRNA sequencing (dual index sequencing/MiSeq). Activated sludge was incubated for 4 weeks (aerated; fed every 3 days with standardized meat extract and samples for sequencing were taken at 7 time points over 28 days to track changes of microbial communities. Silver concentrations were monitored in parallel for dissolved (ultracentrifugation) and total silver concentrations and samples were measured by atomic adsorption spectroscopy (AAS). Analytical results showed in general a low total and dissolved silver concentration in the water phase staying relatively constant over the testing period, suggesting that most of the silver was quickly bound in the organic matrix. Microbial communities showed structural differences between the ionic and the silver nanoparticle treatments and detailed data analysis is currently underway.

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**#1616**

**Genotoxicity of nanoparticles measured by the FADU assay - an automated assay for the assessment of DNA strand breaks in vitro**

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Within the NANOSOLUTIONS project a novel approach measuring DNA strand breaks was applied (FADU assay) successfully. This particular automated DNA damage detection assay is based on progressive DNA unwinding under highly controlled conditions of alkaline pH, time and temperature. A fluorescent dye is used as marker for double stranded DNA and a decrease in the fluorescence intensity indicates an increase in DNA unwinding and consequently a greater number of DNA strand breaks.

JURKAT cells were supplemented with of each engineered nanomaterial (ENM) at three concentrations below IC<sub>50</sub> and close to IC<sub>20</sub> values found in precursory cytotoxicity assays. Controls, untreated and the positive control Etoposide in two concentrations were assayed with every run in parallel in a 96-well format. A short treatment time (30 min) was applied to reduce influences by DNA-repair.

All 31 ENM provided by the NANOSOLUTIONS-consortium have been assayed to evaluate DNA damage effects in comparison to a reference compound in JURKAT cells. Differential DNA damage measures have been identified. Genotoxic effects have been found with 12 of the ENMs at non cytotoxic concentrations. 19 of the ENMs did not show genotoxic effects.

In combination with the robotized execution the assay proved to be fast, reproducible and showed very low susceptibility to interference with the nanoparticles. Genotoxicity assessment by the automated FADU assay in comparison to other methods increases throughput and standardisation.

If required the assay allows the examination of DNA-repair effectivity by comparison of DNA damage measures after short and long treatment times with high resolution. Recently the assay was adapted to human reconstructed skin models which enables trials including the skin barrier function into biological hazard identification.

[Supported by NMP4-LA-2013-309329 NANOSOLUTIONS]

## Investigating the impact of lateral dimensions of graphene oxide flakes on the mesothelial membrane

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Graphene-based materials (GBMs) have been regarded as highly promising materials that can be implemented in a wide range of commercial applications, from inks and spray coatings to electronics and drug delivery. Understanding their toxicological profile is crucial for this implementation to occur. Our group has demonstrated that graphene oxide (GO) of small lateral dimensions (less than 1  $\mu\text{m}$ ) did not trigger significant inflammatory responses after intraperitoneal (IP) injection. In this work, we investigated the impact of GO with large, micrometre-sized lateral dimensions (1 to 20  $\mu\text{m}$ ) using the same route of administration. We hypothesised that large materials will induce a more pronounced cellular response on the mesothelial layer than small ones, potentially similar to what can be seen with long carbon nanotubes. Firstly, we assessed the biodistribution of GO after intraperitoneal injection by SPECT/CT whole body dynamic imaging and showed that a 2D material like GO is able to travel to the diaphragm. In addition, the presence of carbon nanomaterials within the multiple cell layers of the diaphragm was confirmed by Raman mapping of histological sections for the materials tested, namely: long carbon nanotubes, large GO and small GO flakes. Morphological analysis by histology and SEM showed that GO did not induce significant recruitment of granulocytes to the mesothelial layer. Finally, differential cell staining of cells extracted from the peritoneal cavity showed a very limited recruitment of immune cells (not significant) within the cavity (mostly macrophages and neutrophils), which did not vary with the lateral dimensions of the material. These results suggest that 2D materials such as GO of large lateral dimensions (above 5  $\mu\text{m}$ ) do not behave as long carbon nanotubes, possibly because of the higher flexibility of these 2D materials.

## Protein coronation is not fully protecting Beas-2b cells against toxicity of graphene oxide flakes of large lateral dimensions

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Considering the known pulmonary health and safety concerns for different nanomaterials, such as carbon nanotubes, and assuming that the lungs would be the primary organ in contact with the material following the exposure to airborne graphene-related materials (GRMs), there is a need to clarify their impact on cells of the pulmonary system. Given these considerations, the aim of the present study was to determine the role of two key physicochemical parameters (i.e. lateral dimensions and protein corona formation) in the biological response of human lung epithelial cells BEAS-2B to GO exposure. To address these questions, we produced research grade, endotoxin-free graphene oxide (GO) materials of two clearly distinct lateral dimensions – large, micrometer-sized GO (5 – 15 µm) and small, nanometer-sized GO (50 – 200 nm) – using a modified Hummer's method. Importantly, we demonstrated by thorough characterisation that these two materials differed only by their lateral dimensions, allowing us to determine the role of one physicochemical parameter at a time. The role of protein coronation in the initial phase of the interaction between the nanomaterials and cells was addressed by controlling the presence of FBS (0 or 10%) during the first 4 hours of the interaction period. Cytotoxic responses were found to be time- and dose-dependent, especially in absence of FBS for the first 4 h, with the large GO materials inducing significantly higher levels of cell death correlated with elevated ROS production and increased expression of the pro-inflammatory genes. In addition, biological effects induced by small materials were completely alleviated at 24 h in the presence of FBS, while only slightly mitigated for large materials with FBS, confirming the hypothesis that lateral dimensions are a predominant factor of toxicity and inflammation *in vitro*.



## CAN NANOPARTICLE CHARACTERISTICS BE USED TO RANK TOXICITY TO DIFFERENT ORGANISMS?

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Engineered nanoparticles (ENPs) have been shown to cause toxicity in a range of organisms. A key question that remains is whether observed toxic effects are consistent across different organisms and if nanoparticle design properties, such as core chemistry, size and surface coating can be identified to infer the toxicity of other particles by read-across. The aim of this study was to create such a read-across by ranking nanoparticle toxicities to different species (bacteria, algae and nematodes) and identifying common nanoparticle characteristics such as core, size, surface coating and speciation that could be driving the observed effects. The tested ENPs were (1) nine Ag ENPs with varying size (12 – 60 nm), surface coating and speciation, (2) three differently sized (32, 100, 310 nm) polystyrene latex particles with the same surface coating and (3) four TiO<sub>2</sub> ENPs of the same size (25 nm) but with different coatings were tested. In addition to investigate photo activation of the TiO<sub>2</sub> ENPs these particles were also tested under light/dark conditions. To validate if the read across strategy applies for NPs in commercial products testing was extended to include relevant ENPs used in antifouling paints, printing inks and textiles to these species was also assessed. In order to establish the relative toxicity of these ENPs three bacterial species (*Arthrobacter globiformis*, *Janthobacterium lividium* and *Pseudomonas putida*), and the algal species (*Raphidocelis subcapitata*) were exposed to a concentration range of each nanoparticle type and their growth inhibition determined. The effect of the selected ENPs on the reproduction of the nematode *Caenorhabditis elegans* was also assessed. Initial results of these toxicity tests did show matching rankings in the ENP toxicity of the different core chemistries across the organisms (ENP toxicity of Ag > polystyrene latex > TiO<sub>2</sub>). Among the Ag ENPs, Ag<sub>2</sub>S was the least toxic to all tested organisms only little other ENP similarities were found driving toxicity among the remaining ENPs. This was also the case for polystyrene latex particle exposures where the toxicity ranking was also not conserved across the tested species. For TiO<sub>2</sub> ENPs no distinct differences were observed between the surface coatings compared so no rankings could be established for the individual organisms. Overall for each core type no single ENP property (coating, size or speciation) was consistently driving the observed toxicity patterns. For the commercial particles the contribution of the synthesis residues or unknown stabilising agents the supplied nanoparticle dispersion media could not be excluded and need to be investigated in order to remove any confounding effects in their toxicity ranking. Overall a large variation in the toxicities of the ENPs was observed and therefore no single nanoparticle property besides the core could be found to influence ENP toxicity in a manner that was conserved across all tested species.

## THE EFFECTS OF SURFACE COATING ON THE TOXICITY OF CuO ENGINEERED NANOMATERIALS TO EARLY LIFE-STAGE ZEBRAFISH (*DANIO RERIO*)

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A fundamental question in the hazard assessment of engineered nanomaterials (ENMs) is whether surface chemistry plays a significant role in their toxicity. As part of the cross species modelling work in the Nanosolutions project, early life-stage zebrafish (*Danio rerio*) were exposed to CuO ENMs with different surface coatings (thus chemistry). Briefly, fish were exposed in dechlorinated Plymouth tap water ( $27 \pm 1^\circ\text{C}$ ) for 48 h and both lethal and sublethal effects assessed. The treatments consisted of control (no added Cu), soluble Cu control (as  $\text{CuSO}_4$ ), CuO-core (no added coating), CuO-carboxyl ( $\text{COOH}^-$ , negatively charged), CuO-ammonium ( $\text{NH}_4^+$ , positively charged) and CuO-polyethylene glycol (PEG, neutral and hydrophilic). Initial experiments involved exposing the fish to a concentration range of each material ( $0\text{--}100\text{ mg l}^{-1}$  for ENMs and  $0\text{--}1000\text{ }\mu\text{g l}^{-1}$  for Cu as  $\text{CuSO}_4$ ) in order to assess the lethal toxicity. Following this, the mean 48 h  $\text{LC}_{10}$  was calculated for both embryos and larvae and this value ( $900\text{ }\mu\text{g l}^{-1}$ ) used as the nominal concentration for sublethal exposures. The endpoints included biochemistry, morphometrics, bioaccumulation and proteomics (the latter in larvae only). A dose-dependent response was seen in both embryos and larvae exposed to coated ENMs (but not CuO-Core) and  $\text{CuSO}_4$ , with increasing concentrations causing increased mortality. Soluble Cu (as  $\text{CuSO}_4$ ), was more acutely toxic than the ENMs to both of the life-stages tested, whilst embryos were more sensitive to all treatments than the larvae. CuO-Core was the least toxic of the nanomaterials, whilst CuO- $\text{COOH}^-$  was the most toxic to embryos and CuO-PEG to larvae. For embryos, the 48 h  $\text{LC}_{50}$  values were (mean  $\text{mg l}^{-1} \pm \text{SD}$ ); core,  $>100$ ;  $\text{COOH}^-$ ,  $2.45 \pm 1.91$ ; PEG  $2.85 \pm 1.65$ ;  $\text{NH}_4^+$   $3.07 \pm 0.02$ ; Cu as  $\text{CuSO}_4$ ,  $0.27 \pm 0.16$ , whilst the equivalent  $\text{LC}_{50}$  values for the larvae were; core,  $>100$ ;  $\text{COOH}^-$ ,  $8.22 \pm 0.13$ ; PEG,  $5.85 \pm 0.32$ ;  $\text{NH}_4^+$ ,  $8.99 \pm 3.67$ ; Cu as  $\text{CuSO}_4$ ,  $0.48 \pm 0.30$ . Decreased levels of total glutathione were seen in both embryos and larvae exposed to all Cu treatments compared to control animals, but no changes were seen in  $\text{Na}^+/\text{K}^+$ -ATPase activity, apart from an almost two-fold decrease in the CuO  $\text{COOH}^-$  exposed larvae compared to control ( $8.27 \pm 2.42$  and  $4.78 \pm 1.68\text{ Pi }\mu\text{mol mg protein}^{-1}\text{ h}^{-1}$  in the control and  $\text{COOH}^-$  exposed larvae respectively). No biologically significant changes were seen following the morphometric examination (i.e., length, yolk sac volume, muscle block width), however there was some evidence of spinal deformities (lordosis and kyphosis) in CuO- $\text{COOH}^-$  exposed larvae, whilst larvae exposed to CuO-Core also exhibited some eye abnormalities and pericardial oedema. The assessment of bioaccumulation and proteomics are ongoing. In conclusion, the Cu salt was far more toxic than the ENMs, but a clear coating-dependent effect was seen within the ENM treatments. CuO-core was the least toxic, even though it contains the greatest concentration of Cu on an equal mass vs. mass basis.



**Microscopy-based high-throughput assays enable multi-parametric analysis to assess adverse effects of nanomaterials in various cell lines**

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Manufactured nanomaterials (MNM)s selected from a library of different MNMs were tested by different laboratories for toxicity by high-throughput/-content (HT/C) techniques. These MNMs were chosen on the basis of having similar size but different chemistry and coatings. The selected particles include CeO<sub>2</sub>, Ag, TiO<sub>2</sub> (similar size, different chemistry and surface modifications), ZnO (similar size, hydrophobic, hydrophilic) and SiO<sub>2</sub> (similar size, different surface modifications).

The MNMs were tested in different mammalian cell lines to link physico-chemical properties to multiple adverse effects. The cell lines represent different organs such as liver (HepaRG, HepG2), lung (A549), colon (HCT116) and the immune system (RAW264.7). Endpoints such as viable cell count, cell membrane permeability, apoptotic cell death, mitochondrial membrane potential, lysosomal acidification and steatosis have been studied. The outcome of the studies is that the soluble MNMs, Ag and ZnO, were the most toxic in all cell types. TiO<sub>2</sub> and SiO<sub>2</sub> MNMs also triggered toxicity in some, but not all, cell types and the cell-type specific effects of

TiO<sub>2</sub> and SiO<sub>2</sub> NPs were influenced by the specific coating. CeO<sub>2</sub> MNMs were nearly ineffective in our test systems. The differentiated liver cells HepaRG seem to be the most sensitive to MNMs, in particular to titania.

Our results confirm a lot of previous studies using conventional assays providing smaller, fragmented datasets thereby increasing the confidence into the use of various HT/C assays to assess acute toxicity of MNMs. The selective toxicity of metal NPs (Ag, ZnO), which is presumably due to the release of ions, has been confirmed. Also the use of polystyrene NPs as a positive control emerged as a reasonable approach as adverse effects of these MNMs could be detected in all cellular systems.

Whereas most of the investigated MNMs showed no acute toxicity, it became clear that some show adverse effects dependent on the assay and cell line. Hence, it is advised for future studies to use rather a multi-parametric approach such as HT/C screening not to miss signs of acute toxicity. Building on this experience, more refined assays focussing on more sophisticated toxicity read-outs such as inflammation, genotoxicity or disturbance of differentiation and development should be included in a HT/C screening format to broaden the applicability and relevance of current *in vitro* screening approaches. Furthermore, some of the cell type specific effects should be followed up in more detail and might also provide an incentive to address potential adverse effects *in vivo* in the relevant organ.

The authors acknowledge support from the European Commission's 7th Framework Programme project NanoMILE (Contract No. NMP4-LA-2013-310451).

## EFFECTS OF CERIUM OXIDE NANOPARTICLE AEROSOL ON HUMAN LUNG CELLS EXPOSED AT THE AIR-LIQUID-INTERFACE

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The adverse effects of airborne particles are frequently studied by dispersing (collected) particles in culture medium and exposing lung cells under submerged conditions. However, this method does not represent the actual process in the human lung. It even changes the properties of the investigated particles. Particle exposure at the air-liquid interface can eliminate these disadvantages, but requires a well-engineered system to guarantee reproducible conditions (Paur et al., 2011).

Therefore, an advanced automated system for the exposure of cells to nanomaterial aerosols at the air-liquid interface was developed by the Karlsruhe Institute of Technology KIT and VITROCELL® Systems (Mülhopt et al., 2016). Airborne nanoparticles pass a size selective inlet and a conditioning reactor for flow-, temperature- and humidity-control before they are applied to the cells. The deposited particle mass is monitored by a quartz microbalance. The particle dose can be enhanced by an electrostatic field. An internal negative control using humidified synthetic air is implemented. A standard exposure protocol is executed automatically and all settings are controlled and the data are logged.

Dispersions of cerium oxide and two cerium oxides doped with 27 and 78 % of zirconium were synthesised by Promethean (Birmingham, UK) and aerosolised for ALI investigations. CeO<sub>2</sub> was chosen due to the fact that it can cycle between two redox states, Ce<sup>3+</sup> and Ce<sup>4+</sup>, which endows this nanomaterial with catalytic properties, and suggest a mechanism of activity based on oxidative stress. Doping with Zr was used to alter the redox activity. The use of CeO<sub>2</sub> nanoparticles in vehicle catalysts makes it relevant for exposure via inhalation.

For the ALI exposure a suspension of CeO<sub>2</sub> and redox-modified CeO<sub>2</sub> nanoparticles (primary particle size 20 nm) was aerosolized according the VDI guideline 3491 using a two phase nozzle with clean air. The dried aerosol was guided into the conditioning reactor and the particle size distribution was determined by Scanning Mobility Particle Sizer. At KIT A549 human lung cells and at RIVM human bronchiolar epithelial cells (16HBE) in co-culture with endothelial cells (HUVEC) were exposed to the aerosol for 4 hours and analysed for viability and release of cytokines.

In both labs the CeO<sub>2</sub> as well as the redox-modified CeO<sub>2</sub> nanoparticles slightly enhanced LDH release after 4h exposure at a high dose but did not induce IL-8 release after 24 hours submerged post-incubation at KIT. No significant differences between the CeO<sub>2</sub> variants were observed with respect to their toxic properties in both labs suggesting there is no redox-related effect between the tested particles.

The authors acknowledge support from the European Commission's 7th Framework Programme project NanoMILE (Contract No. NMP4-LA-2013-310451).

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## Inflammatory Effects of CuO Nanomaterials in a Murine Model of Asthma

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Engineered nanomaterials (ENM) provide considerable benefits for many sectors of life, however, due to the rapid development of great number of materials, it is relevant to understand how they affect health. Effects of ENM have been broadly studied under normal health conditions but there is insufficient data about their impact on vulnerable populations including people with asthma. The chronic lung disease has become common affecting 300 million people worldwide. The aim of this study was to investigate how uncoated and surface modified CuO nanomaterials affect allergic airway inflammation in a murine model of ovalbumin (OVA)-induced asthma.

Mice were intraperitoneally immunized during the first sensitization period with a mixture of an allergen, ovalbumin (OVA) and an adjuvant, Alum. After 10-day recovery, mice received saline or OVA with or without dispersed CuO materials *via* oropharyngeal aspiration on 4 consecutive days. 24 h later blood and lung samples were collected for various analyses.

Quantitative evaluation of inflammatory cells in BAL showed that all CuO materials (core, -COOH, -NH<sub>3</sub><sup>+</sup>, -PEG) triggered dose-dependent increase in the number of macrophages in the airways of OVA-challenged (asthmatic) mice. The materials induced the influx of neutrophils into the lungs that was most severe in response to CuO-NH<sub>3</sub><sup>+</sup>. Administration of core CuO, CuO-NH<sub>3</sub><sup>+</sup> or CuO-PEG caused significantly increased migration of eosinophils in comparison with OVA-challenged control mice. Eosinophil recruitment was mildly suppressed when mice received CuO-COOH. Significant elevation in lymphocyte numbers was observed upon exposure to each CuO material. Histological assessment of H&E-stained lung sections revealed that all tested materials induced qualitatively similar features in the lung tissue, including macrophage rich areas, neutrophil infiltration and presence of nuclear dust. The effects were accompanied by eosinophilia which is a common sign of asthma. PAS-stained lung sections showed that goblet cell activation was mildly suppressed by CuO materials. No notable changes were seen in the serum levels of OVA-specific IgE after CuO treatments.

Results of this study provide knowledge about how nano-sized CuO modulates allergen-induced asthma. Furthermore, a better understanding how different surface functionalizations influence CuO-triggered responses will be obtained.

The research leading to these results receives funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement number [309329].





## EFFECTS OF SiO<sub>2</sub> AND CeO<sub>2</sub> NANOPARTICLES ON ALZHEIMER-LIKE PATHOLOGY IN MICE AFTER 3 AND 14 WEEKS ORAL EXPOSURE

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There is an increasing concern about neurotoxic and neurodegenerative effects of engineered nanoparticles. We have investigated the effects of oral exposure to SiO<sub>2</sub> and CeO<sub>2</sub> nanoparticles on Alzheimer's Disease (AD)-like pathology in 5xFAD transgenic mice and their C57BL/6J wildtype littermates.

The mice were exposed *ad libitum* for 3 or 14 weeks to control feed pellets, or pellets enriched with either amorphous fumed SiO<sub>2</sub> (7 nm) or CeO<sub>2</sub> (NM-212, EU-JRC repository) at 1 mg/g and 10 mg/g feed. Following exposure, the mice were investigated for various AD-related features, including altered behaviour (i.e. string suspension test, X-maze, open field test), brain tissue homogenate levels of Amyloid- $\beta$  (A $\beta$ ), and formation of A $\beta$ -plaques in hippocampal and cortical brain regions.

No major behaviour impairments could be observed in association with nanoparticle exposure in the transgenic as well as the wildtype mice. Treatment-related variations in A $\beta$ 40 and A $\beta$ 42 protein levels could be detected in the brain homogenates of the mice from both genetic backgrounds. However, immunohistochemical analysis revealed no accelerated formation of A $\beta$  plaques in the nanoparticle-fed 5xFAD mice.

The findings from present study suggest that long-term oral exposure to SiO<sub>2</sub> or CeO<sub>2</sub> nanoparticles has no major adverse health impact on the central nervous system, specifically regarding the development or progression of the neurodegenerative Alzheimer's disease.

Acknowledgment: The work leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° NMP4-LA-2013-310451 and the German Federal Ministry of Education and Research (BMBF/ZonMW project N3rvousSystem).



## NANOMATERIAL GENOTOXICITY: THE CASE OF SILVER NANOPARTICLES

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Although progress has been made in the evaluation of the genotoxicity of nanomaterial (NM), the mechanisms behind this effect is still not well understood. Based on the NM library of Nanomile and using traditional assays and High Content analysis (HCA), the aims of this study were i) to classify NMs with respect to their genotoxic potential, ii) to identify the physico-chemical parameters that would determine NM genotoxicity, and iii) for the NMs causing significant damage to DNA, to delve deeper in the mechanisms behind this effect. Only TiO<sub>2</sub>-NPs, Ag-NPs and MWCNT showed significant genotoxicity.

Ag-NPs induced oxidative damage to DNA, i.e., oxidized bases and DNA strand breaks. The intensity of DNA damage depended on Ag-NP surface coating with NM300K causing more intense damage than PVP-coated Ag-NPs. X-ray absorption spectroscopy analyses showed that Ag-NPs progressively dissolved inside the cells; NM300K dissolved more rapidly than PVP-coated Ag-NPs. The released Ag ions interacted with thiol-containing ligands. The structure around Ag atoms was that of AgS<sub>2</sub> and AgS<sub>3</sub>, with a Ag...S bond distance of 2.47 Å, which is consistent with complexation with –SH containing proteins, such as metallothioneins and Zn-finger proteins. Transmission electron microscopy, energy-dispersive spectroscopy and micro-X-ray fluorescence microscopy proved that Ag deposited in cytoplasmic vesicles together with sulfur atoms, both as spherical nanoparticles and as sheet-like structures. At the cellular level, dysregulation of the redox balance was observed in exposed cells, i.e., increased GSH level and decreased expression of genes encoding anti-oxidant enzymes. Metallothionein gene expression was upregulated. Accumulation of damage to DNA and cell cycle blockade in the G2 phase were observed, together with significant reduction of DNA repair capacity and decrease of DNA repair gene expression.

In summary Ag-NP surface coating, and thereby Ag-NP dissolution, was the primary parameter that determined their genotoxic impact. Upon dissolution, Ag-NPs would inactivate proteins that are dedicated to cell defence, and therefore cause primary indirect genotoxicity.

#1551

**A HIGHTHROUGHPUT APPROACH TO UNDERSTAND THE TOXICITY MECHANISM OF A SUPRAMOLECULAR BASED NANOPARTICLE WITH COMBINATIONAL CHEMOTHERPY AND PHOTODYNAMIC THERAPY EFFECT**

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A metallacage consist of Pt based chemotherapeutic agent and porphyrin based photosensitizer was prepared, using multicomponent coordination-driven self-assembly. The formation of metallacage-loaded nanoparticles (MNPs) occurs when the assembly is treated with two variants of a 1,2-distearoyl-phosphatidylethanolamine/polyethylene glycol conjugate, mPEG-DSPE and cRGD-PEG-DSPE, the former terminating in a methyl group and the latter in a tumor targeting peptide. This combination endows the resultant MNPs with excellent stability and targeting ability, specifically enabling selective delivery of the metallacages to cancer cells that overexpress integrins via receptor-mediated endocytosis. *In vitro* and *in vivo* studies (mouse model) indicate that the MNPs are more chemotherapeutic active and show low systematic toxicity while also possessing photodynamic therapy ability under a 630 nm laser.

To understand host response to systematic administrated nanoparticles with combinational therapeutic effect, colorectal tumour gene expression profiles in mice were generated using a GeneChip PrimeView Human Gene expression array. These arrays contain more than 50,000 probes housing over 47,000 transcripts and variants, which reproduce more than 38,000 genes that are plotted through RefSeq or via UniGene annotation. For gene expression heat maps, the average expression across three independent replicates in each condition based on the probes with the maximum signal intensity per gene was calculated.

The data were analysed by unsupervised hierarchical clustering, which showed that the both treatment groups had distinct gene expression profiles. Using a threefold change relative to the control group as a benchmark for differential expression, numerous genes were derived from the different therapeutic modalities. For the clustering of differentially expressed genes, a total of 18,057 genes were analyzed and an absolute  $\log_2$  fold change of  $>1$  with a p value of  $<0.05$

to define the differentially expressed gene sets was used. This analysis enables pinpointing distinct gene expression fluctuations related to chemotherapy and/or phototherapy with several clusters of genes that were only suppressed or induced in single treatment or combinational treatment.

Our profiling analysis allowed us to identify potential intermediaries of tumour response to therapy. By employing a network analysis methodology, we could classify and rank numerous cores that were related to response to therapy. Specifically, whole-gene expression analysis of tumours from treated mice suggests that phototherapy treatment leads to the repression and induction of a higher number of genes and variants when compared with chemotherapy. Molecular pathways that are mainly regulated by genes controlling cytoskeleton remodelling and intracellular transport were 'turned on' in response to phototherapy, whereas transcription and metabolism mechanisms were stimulated following chemotherapy.

The wide genome array assays from tumours in response to each of the single therapeutic modalities and especially to the combinational therapy provide potential biomarkers for drug/phototherapy in clinical application. The biomarkers identified herein via quantitative gene expression analysis may be used in the future to better design materials that can target specific genes/pathways to achieve better clinical outcome.

#1555

## PRECISION-CUT TISSUE SLICES: A POTENTIAL *EX-VIVO* MODEL FOR NANOSAFETY STUDIES?

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Nanomaterials have the capacity to enter and distribute within organisms, often without proof of export<sup>1</sup>; because of their increased use in nanotechnology and nanomedicine applications, it is of key importance to assess the potential impact of these materials on cells and organisms.

Precision-Cut Tissue Slices (PCTS) represent an interesting *ex vivo*-model already used for pharmacotoxicological studies and drug metabolism<sup>2</sup>: the maintained cellular complexity of the tissue can constitute a major advantage in respect to simpler cell cultures. In addition, it is possible to prepare slices from diverse species, including from human tissue (for instance from surgical waste) and different organs. Furthermore, PCTS may significantly contribute to the reduction of *in-vivo* studies in accordance with the 3Rs – replacement, reduction and refinement. PCTS may therefore have potential also as a novel tool to support nanosafety studies.

Within this context, as a first step, we aim to investigate whether PCTS can be used as an additional model for nanosafety assessment.

For this purpose, we are investigating uptake and distribution of model nanoparticles (NPs) such as fluorescently labeled carboxylate polystyrene (PS-COOH) and silica (SiO<sub>2</sub>). NPs are added to the PCTS in relevant biological media, such as medium containing serum in order to mimic realistic exposure scenarios<sup>3</sup>. Confocal fluorescence imaging is used in order to determine whether NPs enter cells, how deep they can travel within the tissue, in which cell types they accumulate and their intracellular localization. The outcomes are compared to what is observed *in vivo* for similar systems.

In addition, well-known toxic nanoparticles such as amino-modified polystyrene (PS-NH<sub>2</sub>) NPs<sup>4</sup> are used in order to define whether it is possible to translate to the PCTS the cytotoxic effects observed for the same materials in *in-vitro* studies.



Extensive optimisation of different steps from NP exposure to sample preparation has been performed. Despite a large adsorption of NPs on the outer layer of the tissue, uptake of NPs is clearly visible inside the section and in specific cell types.

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**#1504**

**A CONCEPT FOR DECISION MAKING ON INNOVATIVE IRON OXIDE  
NANOPARTICLES SELECTION FOR A SUSTAINABLE APPLICATION IN  
DIAGNOSTICS**

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In responsibility for future generations our world has to deal with e.g. increasing global population, environmental protection and resource scarcity. Therefore, it is indispensable to foster sustainable innovation and development. The European Union defined six key enabling technologies (KET: Nanotechnology, Nanoelectronics, Photonics, Industrial Biotechnology, Advanced Materials, Advanced Manufacturing) with an important impact on a future sustainable and inclusive growth of European industries. In case of nanotechnology, high potentials could be already shown in e.g. transport, construction, energy or medicine and pharmacy. However, critical questions, e.g., concerning safety and environmental protection have to be taken into account as well to allow informed strategic decisions.

In this context, the collaborative BMBF-project NanoBEL (biological elimination of complex diagnostic nanoparticles) aims to select modified organic core-shell iron oxide nanoparticles to analyze the long term toxicological effects and the potential applicability in diagnostics (e.g.

contrast agent in magnetic resonance imaging). The selection of formulations follows a multi-staged safe-by-design-approach (SbD). Characterization of physicochemical properties in water (SbD-stage 1) is followed by SbD-stage 2 which includes *in-vitro*-toxicity-testing, stability and degradability in biological media to estimate the potential biological impact. Based on those results, a selection of formulations for long term *in-vivo*-analysis is taken. In parallel, the potential of the favored formulations in terms of sustainable and safe-by-design medical products will be analyzed in a holistic assessment combining methodologies of qualitative risk assessment (e.g. swiss precautionary matrix, Stoffenmanager Nano 1.0, NanoRiskCat), life cycle assessment and technical feasibility in a multi-criteria decision analysis. This overall concept considers aspects such as technology scale-up concepts, resource depletion, energy consumption, environmental impacts, costs and human health risks during preparation, medical use up to the end of life (waste treatment, environmental fate) (SbD-stage 3).

Finally, the potential of modified iron oxide nanoparticles in diagnostics will be critically evaluated compared to alternative gadolinium-based contrast agents taken as baseline.

The project is funded by the German Ministry of Education and Science (BMBF), grant 03XP0003A-H.

## DEVELOPMENT OF OPTIMAL SYNTHESIS STRATEGIES FOR THE REDUCTION OF NANOTECHNOLOGY ASSOCIATED RISKS

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The growing interest in the field of nanotechnology leads to an increase of the number of nanoparticles (NPs) producers and users, making indispensable the promotion of safe strategies in the development and management of nanomaterials nowadays.<sup>(1)</sup>

The need to introduce in society commercial products that are at the same time useful and safe should start from the very beginning of their conception establishing safer by design synthetic approaches.

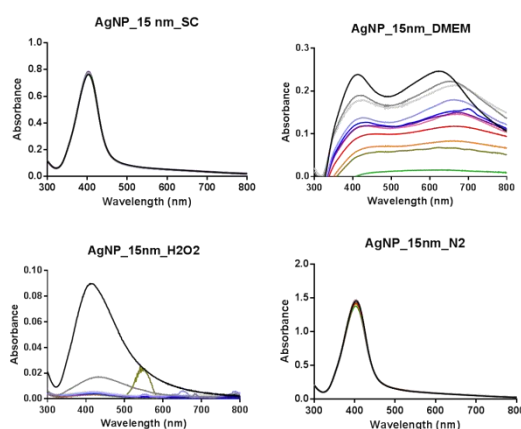
One of the main objectives of making secure nanomaterials (NMs) is to ensure that their desired functional properties do not change with the introduction of different synthetic strategies. Among these strategies will be included: morphology (size and shape), surface state (stability, hydrophilicity, charge) and reactivity (redox potential, corrodibility, catalytic activity). These parameters could reduce exposure to NPs and their hazards while maintaining the desired properties of the NPs in a determined application.

In our synthetic group we have used three general safer-by-design strategies regarding the re-design of silver NPs (owing to the well known toxicity to several biological organisms):<sup>(2)</sup>

1. **Reducing the toxicity** of the materials. For instance, modifications in the size and shape could reduce the toxicity of the NMs in a specific biological system. We have seen that as NP size increases, the NP becomes more chemically stable but it is more difficult to maintain it in suspension.
2. **Reducing the release** of NMs from the matrix during their life cycle. For this purpose it is needed to coat the NMs with different ligands. For instance, applying a surface coating (such as polyvinylpyrrolidone, PVP) could enhance the adhesion with the matrix compared with the naked surface. Thus the release of NMs from the matrix can be controlled.
3. **Reducing persistence of NMs.** Development of biodegradable NMs under certain temperature or oxidative conditions (**Fig. 1**). The addition of protective agents to avoid the spontaneous transformation of NMs, for instance using albuminization strategy. Or studying the transformation of NMs and their corresponding toxicity by changing the NM composition, such as Ag and Ag<sub>2</sub>S in the presence of S<sup>2-</sup> ions.

In summary, we focus on surface state, which can protect the NP from corrosion (avoiding the leaching of toxic ions), which can protect the surrounding molecules from unwanted catalytic degradation due to the NP surface, which can control interaction with biological moieties, as

proteins or phospholipids (cell membranes), and therefore exposure and hazard to living systems.



**Fig 1.** Corrosion of 15 nm silver NPs. Evolution of UV-Vis spectra of nanoparticle degradation under oxidative conditions. The outcomes reflected in this work were performed under the ongoing GUIDEnano project funded by European Commission's Framework Programme (FP7/2007-2013) under grant agreement №604387.

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## NANOSTREEM: STRATEGIES FOR SAFETY ASSESSMENT IN ADVANCED INTEGRATED CIRCUITS MANUFACTURING

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Nanoelectronics industry is a key enabler of global economic growth and development. Within Europe, the nanoelectronics industry is estimated to have an economic value of €30 billion, and is responsible for 200 000 direct and more than 800 000 induced jobs. Nanoelectronics relies on multiple semiconductor processes resulting in patterning of macroscopic objects (silicon wafers) at nanoscale level. Overall, the semiconductor industry can be considered predominantly as a user of nanomaterials in integrated circuits fabrication. The rapid pace of progress in the semiconductor manufacturing dictated by the Moore's economic law also introduces a variety of novel nano-structured materials having poorly understood hazardous properties. New materials, including variety of nanoparticles are constantly introduced in the development of advanced technology nodes. This is recognized as a shared concern by the industry; therefore a number of smaller scale targeted process safety and occupational health monitoring campaigns have been conducted.

Understanding properties of engineered materials and how they affect biological systems, human health, and the environment is a relatively new area of scientific study which requires long term efforts, often coming long after a product is on the market. Such a situation presents a challenge for both mitigation of the occupational and environmental risks and the overall risk governance and policymaking. As a step in the direction of addressing this challenge 14 industrial and academic institutions from 6 EU member states initiated a collaborative project named "**Nanomaterials: Strategies for safety Assessment in Advanced Integrated Circuits Manufacturing**". NanoStreeM ([www.nanostreem.eu](http://www.nanostreem.eu)) is a project funded under Horizon 2020 EU Framework Program for Research and Innovation<sup>13</sup>.

The overall goal of the project is to support and coordinate activities in relation to safety and occupational aspects of nanomaterial use with a specific focus on the semiconductor industry as a use case. The general objectives of the project are

- (i) to build inventories of materials, research topics and directions relevant for nanomaterial use and exposure in nano- electronics manufacturing (Work package 1);
- (ii) to identify gaps in knowledge and methodologies to assess the risk of engineered nanomaterials used in semiconductor manufacturing or incidentally released as by-products of the manufacturing process (Work packages 2 and 3);

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<sup>13</sup> The project receives funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 688194.

- (iii) to apply obtained results for better training, management and governance of the risks related to engineered nanomaterials and industrial processes (Work packages 4 and 5).

The current phase of the project focuses on establishing trajectories of nanomaterials in semiconductor production facilities, identifying operations of concern and air sampling techniques suitable for demonstration of nanoparticle emissions in clean rooms.

**NPS RELEASE STUDY TURNED TOWARDS THE SAFER BY DESIGN  
APPROACH – DIFFERENT CASE STUDIES INVESTIGATED IN THE  
GUIDENANO EUROPEAN PROJECT**

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Within the frame of the European GUIDENANO project, several industrial partners work with scientists in order to assess the release of NanoParticles (NPs) integrated in several NanoMaterials (NMs) and to find some ways to overcome the release through safer by design formulations. The presentation will focus on three different case studies: nanoTiO<sub>2</sub> photocatalytic tiles, nanoZnO antifouling paints and food packaging coated nanocellulose. Climatic ageing and mechanical solicitations were made on photocatalytic tiles and on antifouling paints. In the case of the nanocellulose material, only abrasion experiments were investigated. Results obtained by Fast Mobility Particle Size (FMPS) on abraded NMs are gathered on Figure 1.

- The climatic ageing of photocatalytic tiles studied by X-Ray Photoelectron Spectroscopy (XPS) evidenced the degradation of their extreme surface, leading to an accumulation of TiO<sub>2</sub> NPs after 500h. Between 500h and 1000h, water spray caused the leaching of these TiO<sub>2</sub> NPs which were observed in the run-off waters by Scanning Transmission Electron Microscopy (STEM). Abrasion experiments performed on safer by design photocatalytic tiles (TiO<sub>2</sub> NPs with SiO<sub>2</sub> coating) showed an increase of NPs release compared to the first generation of tiles. The less releasing tile is found to be the 1st generation ones. A new safer by design formulation is under investigation.

- Globic and Olympic antifouling paints were aged and abraded. It was measured by FMPS during abrasion that the Globic paint released more particles. It is the expected effect for an antifouling material. This paint is then the best in term of antifouling effect. However, the critical



point is the presence of a great amount of NPs in the aerosol. New formulations of Globic paint are under investigation in order to reduce the emission of NPs while maintaining the antifouling effect.

- The food packaging investigated was composed of a cellulose substrate coated with a thin layer of nanocellulose. To study the release from this “stack”, a labelling of the nanocellulose was developed to distinguish NPs coming from cellulose substrate from those coming from the nanocellulose coating. Abrasion results indicated that the nanocellulose deposited on the cellulose substrate do not increase the number of aerosolized NPs during abrasion compared to the substrate alone. Moreover, the labelling of the nanocellulose allowed us to observe by Scanning Electron Microscopy (SEM) labelled NPs emitted from the nanocellulose coating. With a slight friction, NPs emitted from the

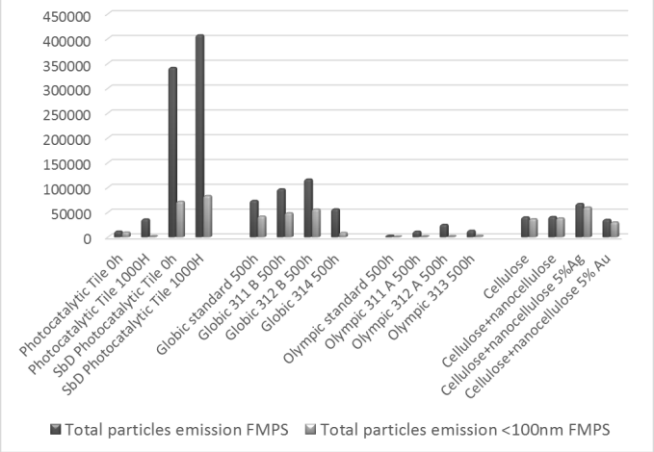


Figure 1: Total particle and NPs number measured by FMPS during abrasion process of photocatalytic tiles, antifouling paints and food packaging.

## THE SURFACE PROPERTIES OF NANOMATERIALS DOMINATE CELLULAR UPTAKE AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Uptake of nanomaterials may bring some unexpected effects such as cytotoxicity and alternation of cell functions. The impact is largely dependent on physicochemical properties of NPs such as size, agglomeration tendency, shape, surface chemistry and protein corona as well as cell type. Stem cells have been widely studied for their peculiar capability to self-replicate and differentiate into cells of specific lineages. Therefore, it would be of interest to study the interaction and subsequent influence on stem cells differentiation of engineered nanomaterials. In this study, several metal oxide nanoparticles such as copper oxide, iron oxide and titanium oxide were used as representatives of metal oxide nanomaterials to study their potential toxicity to mesenchymal stem cells, especially their impact on the functions related to differentiation.

In the first example, two different kinds of iron oxide nanoparticles, with a similar size (~10 nm) but different surface coatings, i.e. pristine particles (P-NPs) and citric acid modified particles (C-NPs) were used. After being incubated with rat mesenchymal stem cells (MSCs) for 14 d, both types of NPs showed similar cell uptake kinetics and final intracellular iron content, i.e. 53.3 pg per cell for P-NPs and 59.9 pg per cell for C-NPs, and minimal cytotoxicity at a concentration below 100 µg/mL. The adipogenic differentiation potential of MSCs was unaltered regardless of the NP types, and the P-NPs did not have an obvious impact on the osteogenic differentiation potential of MSCs. The osteogenic differentiation potential of the MSCs, however, was significantly impaired by incubation with the C-NPs, as evidenced by significantly reduced expression of osteogenic markers, namely collagen type I (COL) and osteocalcin (OCN) and calcium deposition.

In the second example, four kinds of TiO<sub>2</sub> nanorods (TiO<sub>2</sub> NRs) with a similar aspect ratio but different surface functional groups, i.e. amines (-NH<sub>2</sub>), carboxyl groups (-COOH) and poly(ethylene glycol) (-PEG), were used to study their interaction with MSCs. The aspect ratios of the TiO<sub>2</sub> NRs were measured (50 to 65 nm in length and 8 nm in width) under TEM. The MSCs ingested larger amounts of TiO<sub>2</sub>-Core NRs and TiO<sub>2</sub>-NH<sub>2</sub> NRs than those of TiO<sub>2</sub>-COOH NRs and TiO<sub>2</sub>-PEG NRs, with a similar intracellular distribution pattern. TiO<sub>2</sub>-Core NRs induced the highest cytotoxicity as a result of the highest intracellular level of reactive oxygen species

(ROS), which was lowered upon surface functionalization. All of the TiO<sub>2</sub> NRs did not show obvious influence on the adipogenic differentiation potential of the MSCs. But the TiO<sub>2</sub>-COOH NRs showed significantly impairment on the osteogenic differentiation behaviors. The influence of TiO<sub>2</sub> NRs on the osteogenic differentiation of the MSCs was further quantitatively studied by the expressions of osteogenic markers (COL and OCN), in both gene and protein levels. The results confirmed the strongest hindrance of osteogenic differentiation of the MSCs by TiO<sub>2</sub>-COOH NRs, due to up-regulation of transforming growth factor beta 1 (TGF-β1) and fibroblast growth factors (FGF-2).

All these results provide new information that the differentiation potential of the MSCs can be influenced by the presence of nanomaterials with different surface functionality, suggesting the necessity of careful analysis of the biological impact of nanomaterials.

#### Acknowledgement

We thank all the NANOSOLUTION partners for their stimulating discussion and helpful suggestions. This work is financially supported by the Frame Work program 7 of the European Commission (Nanosolutions 309329, BRASINOEU 318916) and the Natural Science Foundation of China (51120135001).

## CHARACTERIZATION AND PROPERTIES OF MODIFIED CuO-NPs IN ENVIRONMENTAL AND BIOLOGICAL MEDIA

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Nowadays engineered nanomaterials (ENM) are used for different applications, e.g. in cosmetics, textiles, coatings and paints, that can lead to their release in the environment.<sup>1</sup> There is a strong likelihood that human exposure potential and biological activity of nanoparticles will depend on key physicochemical properties that represent the so-called exposure and hazard determinants.<sup>2</sup> The investigation of interactions between ENM and the surrounding media, e.g. dissolution, aggregation and surface ligand adsorption, are of great importance for supporting the risk and safety assessment of these materials.<sup>3</sup>

Within the EU-FP7 SUN project, the present study aims to provide safer by design copper oxide engineered nanoparticles (NP), by modifying their surface chemistry with non-hazardous organic compounds. Colloidal stability, surface charge and ion release of both pristine NP and modified samples dispersed in different media relevant for toxicological and ecotoxicological testing are investigated. From the combination of different analytical techniques, such as dynamic and electrophoretic light scattering (DLS and ELS), centrifugal separation analysis, infrared spectroscopy (IR), transmission and scanning electron microscopy (TEM and SEM) and inductively coupled plasma mass spectrometry (ICP-MS), a comprehensive characterization of these new modified ENM have been performed. In detail, the behavior of the modified ENM in environmental (i.e. artificial fresh and marine waters) and biological media (i.e. phosphate buffer solution and Dulbecco's Modified Eagle Medium) has been investigated by DLS and analytical centrifuge. The obtained results allow to estimate the qualitative and quantitative stability of the tested nano-dispersions over time, and further significant information for a better correlation with the results from toxicity and ecotoxicity studies. The approach presented here can greatly support the development of the environmental and human health risk assessment and management of ENM, within a safer by (molecular) design approach.

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Acknowledgement - The authors gratefully acknowledge support from the European Union Seventh Framework Programme [FP7/2007-2013] under ECGA No. 604305 “SUN” and from University Ca’ Foscari of Venice.

## PULMONARY TOXICITY OF SURFACE MODIFIED COPPER OXIDE NANOPARTICLES

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Copper oxide nanoparticles (CuO NPs) have various industrial applications e.g. in antimicrobial products, inks and coatings in food packaging. It has been shown that inhalation of CuO NPs lead to a dose-dependent pulmonary toxicity in rats (Gosens *et al.* Nanotoxicology 2016). In this study, 10 nm CuO NPs were modified with either a polyethylenimine (PEI) or ascorbate coating (ASC). We hypothesized, based on in vitro study results using RAW264.7 cell macrophage-like cells, that negatively charged ASC coated CuO NPs could contribute to a safer design by inducing less pulmonary toxicity compared to a positively charged PEI coated NP. Rats were exposed nose-only to a fixed exposure concentration of ASC and PEI CuO NP at 5 consecutive days. By varying the exposure duration, 3 hour-concentration equivalents of 0, 0.8, 2.3, 7.5 and 21.9 mg/m<sup>3</sup> for ASC and 0.6, 1.8, 6, and 17.3 mg/m<sup>3</sup> for PEI were generated. After exposure, on day 6, and on day 27, pulmonary toxicity markers in bronchoalveolar lavage fluid were analyzed and benchmark dose response analysis was performed. Lung histology revealed interstitial/alveolar inflammation and hypertrophy/hyperplasia of bronchioles/alveoli with accompanying alveolar (cellular) debris in the lung and paracortical histiocytosis in the mediastinal lymph node. Although all treatment groups were affected, the findings starting at a dose of 1.8 mg/ m<sup>3</sup> CuO-PEI and 2.3 mg/m<sup>3</sup> CuO-ASC were considered to be adverse, based on its nature and severity. BALF analysis supported the finding of a dose dependent pulmonary inflammation and cell damage. No differences in the initial toxic potency between the two coatings could be established. However, in animals exposed to PEI coated NPs, a residue of inflammatory cell accumulation remains in the lung at day 27. After exposure to ASC modified CuO NP, there appears to be no inflammatory cell accumulation at day 27 for any exposure concentration. This suggests that during the recovery period, the ASC coating may have provided some protection, preventing the inflammatory effects to persist to day 27.

In conclusion, initially similar pulmonary responses were found for both negatively and positively charged surface modified CuO NPs in vivo, that were not markedly different compared to the

pristine CuO NPs. ASC coating seems to protect against persisting inflammatory effects after a recovery period of 3 weeks.

## IMPLICATIONS OF REDOX ACTIVITY OF NANOPARTICLES ON IMMUNOLOGICAL PATHWAYS *IN VITRO*

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Understanding of hazardous physicochemical properties of nanomaterials is essential for the safe by design approach. The redox activity of nanoparticles (NPs) may adversely influence the immunological pathways and cause inflammatory reactions via oxidative stress through the production of reactive oxygen species. In view of the inflammatory effects observed in the lung after inhalation of NPs *in vivo*, we investigated the effects of a set of redox modified NPs on macrophage inflammasome activation and dendritic cell (DC) maturation *in vitro*. NPs were redox modified via zirconium (Zr) doping of cerium dioxide (CeO<sub>2</sub>) NPs and iron (Fe) doping of cobalt oxide (Co<sub>3</sub>O<sub>4</sub>) NPs. Inflammasome activation was investigated in THP-1 human monocytes that were differentiated to macrophages by phorbol myristate acetate (PMA). After exposure of THP-1 derived macrophages to NPs for 48 hours, cell viability and IL-1 $\beta$  production were measured. Most NPs caused a dose-related decrease in cell viability, but only some NPs also caused an increase in IL-1 $\beta$  production, indicating inflammasome activation. For most NPs a doping-related effect on cell viability was observed, but only for some Fe-doped Co<sub>3</sub>O<sub>4</sub> NPs a doping-related effect on inflammasome activation was observed. DC maturation was studied using human peripheral blood mononuclear cells that were cultured for 6 days with GM-CSF and IL-4 to obtain immature DCs. The immature DCs were exposed to NPs for 48 hours, which did not lead to a major decrease in cell viability for most NPs. Only a few Fe-doped Co<sub>3</sub>O<sub>4</sub> NPs caused a dose-related increase in IL-12p40 production, indicating DC maturation. Fe<sub>3</sub>O<sub>4</sub>-doping seemed to stimulate, while Fe<sub>2</sub>O<sub>3</sub>-doping seemed to reduce DC maturation.

Acknowledgments: The work leading to these results has received funding from the European Union's Seventh Framework Programme for research, technology development and demonstration under grant agreement n° 310451 (NanoMILE) and the Netherlands Food and Consumer Product Safety Authority.





## THE INFLUENCE OF REDOX ACTIVITY OF INHALED NANO-SIZED CERIUM DIOXIDE ON RESPIRATORY, IMMUNE AND CARDIOVASCULAR EFFECTS IN MULTIPLE MOUSE MODELS

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As part of the safer by design strategy, knowledge on the influence of the physicochemical characteristics of nanomaterials on their toxic potential and the underlying biological mechanisms of action is needed. Here we assess the influence of redox activity using a series of zirconium (Zr) doped cerium dioxide nanoparticles (CeO<sub>2</sub> NPs) on respiratory, immune and cardiovascular effects in mice following subacute inhalation.

Healthy (C57BL/6J) mice, mice prone to cardiovascular disease (Western-diet fed ApoE<sup>-/-</sup>) and ovalbumin (OVA) sensitised BALB/c mice were used. All mice were either exposed to clean air or CeO<sub>2</sub> NPs with 0, 27 or 78% Zr-doping, by inhalation over a 4-week period (4 mg/m<sup>3</sup> for 3 h/day, 5 days/week). BALB/c mice were also challenged with OVA two weeks post-exposure to evaluate the adjuvant potency of the CeO<sub>2</sub> NPs. In the other mouse models, effects were assessed four weeks post-exposure to coincide with the formation of complex atherosclerotic plaques as well as plaque formation in the brain.

CeO<sub>2</sub> NP exposure had no major effects on clinical parameters, mortality, body weight, organ weights, blood cell counts (total and cell differentials) and the bronchoalveolar lavage fluid. CeO<sub>2</sub> NP exposure caused modest inflammatory lesions in the lung, which were not different between the various redox modifications investigated. A slight increase in serum OVA-specific IgG1, but not IgE, accompanied with Zr-doping related differences in cytokine production of isolated spleen and lymph node cells observed in CeO<sub>2</sub> NP exposed BALB/c mice, suggested a mild adjuvant effect of CeO<sub>2</sub> NPs. CeO<sub>2</sub> NPs did not alter the size of atherosclerotic plaques in

the brachiocephalic arteries of the ApoE<sup>-/-</sup> mice, although there was a trend towards an increased inflammatory cell content in plaques with increasing Zr content of the NPs.

These findings suggest that Ce<sub>2</sub>O NPs had minimal toxicological effects following subacute inhalation and that redox-modification via Zr-doping of CeO<sub>2</sub> NPs had limited effects on these responses. Further studies with nanomaterials of greater inherent toxicity or a wider range of redox activities are required to fully assess the influence of redox-modification on the toxicity of nanomaterials.

Acknowledgments: The work leading to these results has received funding from the European Union's Seventh Framework Programme for research, technology development and demonstration under grant agreement n° 310451 (NanoMILE) and the Netherlands Food and Consumer Product Safety Authority.

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## IODINE-124 CHEMISORPTION ON METAL NANOPARTICLES WITH ORGANIC COATING FOR *IN VIVO* BIODISTRIBUTION STUDY

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Metal NPs with organic coating are widely used because they combine the properties of the core and the coating. Silver and gold NPs are particularly interesting because of their potential use in imaging. In nanomedicine, for example, the coating can be modified to improve biocompatibility and circulation half-life of NPs, besides conferring targeting properties to the NP. The possibility to follow NPs *in vivo* is important to improve NPs performance and trace their toxicological profile. In this work, we successfully labelled silver and gold NPs with different organic coatings by iodine-124 chemisorption. Pegylated NPs radiolabeling efficiencies were lower respect to the others (about 50% and above 90%, respectively). *In vivo* biodistribution experiments were performed on mice. NPs showed different organs accumulation and kinetics profiles. High accumulation was detected in the thyroid for all NPs excepted for carboxylated Ag NPs that were found mainly in the liver. Gold NPs were cleared mainly through urine as bladder high accumulation suggests. On the contrary, silver NPs accumulated preferentially in liver and stomach.

## THE EFFECT OF A CROSS-LINKED DEXTRAN COATING ON THE POTENTIAL THROMBOGENICITY OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES

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Superparamagnetic iron oxide nanoparticles (SPIONs) are being developed for intravenous injection as contrast agents for medical imaging. However, their potential to promote the thrombogenicity of blood, which could increase the risk of cardiovascular complications in patients, has yet to be fully established. Clinically-applied SPIONs have a modifiable hydrophilic dextran coating to improve particle dispersion, limit leaching of iron and manipulate cellular interactions. Here we hypothesise that SPIONs with a 'conventional' dextran coating promote the thrombogenicity of blood *ex vivo*, an effect that is not induced by SPIONs with a cross-linked dextran coating.

Unmodified SPIONs were synthesised by co-precipitation of FeCl<sub>2</sub> and FeCl<sub>3</sub> in the presence of dextran T10 at 80°C (Nano4Imaging GmbH, Germany). SPIONs were crosslinked with 5% glutaric acid via EDC/NHS chemistry and purified by dialysis to remove unbound reagents. Primary particle size (TEM) was approximately 3-5 nm. Mean particle size in distilled water (DLS) was not significantly different between the two SPIONs (23 ± 3 nm for Unmod, 27 ± 3 nm for X-SPIONs; *p*=0.3). Similarly, there was no significant difference in the zeta-potential of either SPIONs (4.3 ± 0.3 mV for Unmod, 3.1 ± 0.6 mV for X-SPIONs; *p*=0.09).

The thrombogenic effects of SPIONs were tested in a flowing human blood *ex vivo* using a Badimon chamber. Blood was taken from healthy volunteers and SPIONs infused into the blood within the Badimon circuit (volunteers themselves were not exposed) at an effective blood concentration of 300 µg/mL. Exposed blood passes into the Badimon chamber containing 3 sections of porcine aorta with their intimal surface has been removed. Blood clots on the exposed thrombogenic surface of the arterial strip and is quantified histologically, with the area

of the clot being proportional to the thrombogenicity of the blood. Parallel *in vitro* experiments are being performed to determine the role of platelet activation in the action of nanoparticles. Results will be un-blinded to investigators in November 2016.

The Badimon chamber has the advantages of determining blood thrombogenicity in human blood under flowing conditions on a pathophysiologically relevant substrate. Furthermore, the rheological conditions of the Badimon chambers provides conditions that mimic the human coronary circulation, either that of healthy patent arteries coronary arteries, or those that are mildly stenosed. Thus the model provides an excellent system to safely investigate the potential for nanoparticles to promote blood clotting in man, as well as test the ability of surface modifications to minimise the potential unwanted thrombogenic actions of nanoparticles.

*This work was funded by a Seventh Framework Programme (FP7) from the European Commission, ("NanoMILE", NMP4-LA-2013-310451). Studies were approved by local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers.*

## AgNPS CYTOTOXICITY DIFFERENCES RELATED TO PRIMARY SIZE AND FUNCTIONALIZATION

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Safe by design strategies related to nanomaterials are intended to mitigate the potential risk of release to the environment that a specific nanoparticle can exert during its life cycle, without changing the functional purpose for which it was manufactured. One of the objectives of the GUIDEnano project is to develop a strategy to compare and assess the hazard potential of the “exposure relevant forms” resulting from various NM safer-by-design activities.

To perform the present study, several AgNPs were provided by ICN Institute (Spain) and by PlasmaChem GmbH (Germany). The NPs tested were uncoated AgNPs of different sizes (15, 50, 150 nm), functionalized AgNPs (AgO passivation (50 nm), PVP (50 nm), tannic acid (TA) or mercaptosuccinic acid (MSA) coated (both of ~15 nm)) and Ag<sub>2</sub>S transformation product from AgNP (30-40 nm). In vitro assays were developed with the hepatoma cell line RTH-149, derived from rainbow trout (*Onchorrhynchus mykiss*). Cells were exposed during 24 h to the NPs, as well as to their related vehicles and to AgNO<sub>3</sub> as a reference. Cytotoxicity was evaluated using three different assays (Alamar Blue, CFDA-AM and Neutral Red Uptake), testing a range of concentrations up to a maximum of 10 mg/L for AgNPs coated with TA or MSA and of 400 mg/L for the other NPs. Pristine NPs and NPs in the exposure medium were characterized by TEM, DLS and ICP-MS.

Results showed differences in toxicity depending on the NP size with uncoated AgNPs of 50 and 150 nm showing similar toxicity, and higher than those of 15 nm. Regarding functionalized AgNPs, uncoated 50 nm-AgNPs and the same NPs coated with PVP or modified by AgO passivation showed similar toxicity. An increase in toxicity was observed in the AgNP of 15 nm coated with TA or MSA respect to the uncoated NP. Silver sulfide (Ag<sub>2</sub>S) is a common product generated from AgNPs once they are released in the environment and, as confirmed in the present study, it possesses negligible toxicity compared to other materials. To confirm these results another fish cell line (PLHC-1) is being exposed to the same compounds.

Our results do not confirm a direct relationship between small size and higher toxicity due to a higher surface area and related ion release. Functionalization was made to enhance dispersibility (PVP coating), to control oxidation of metal NP (AgO passivation) leading to the mitigation of its corrosion and thus the release of ions, and to enhance the adhesion of NMs on cotton surface (TA and MSA coatings). Our results showed that uncoated AgNPs of 15 and 50 nm were less or equally toxic, respectively, than functionalized NPs.

In conclusion, the safe by design strategies proposed were appropriated for the NPs of 50 nm but not for those of 15 nm in terms of toxicity. However, the balance between toxicity values and environmental concentrations of the modified NPs should be also evaluated in order to reach a final conclusion.

**Acknowledgements** - FP7 project GUIDEnano (agreement nº 604387).

## A METHODOLOGICAL APPROACH FOR THE SAFE DESIGN AND PUTTING INTO SERVICE OF NEW PRODUCTION PROCESSES FOR MANUFACTURING

### CNT-NANO-ENABLED PRODUCTS

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The production of new ENMs and nano-enabled products (NEPs), involves in parallel the design and commissioning of new sustainable manufacturing processes or the modification of existing ones to adapt them to new manufacturing needs. Scaling from laboratory plants to commercial production processes represents a drastic leap, both in terms of technology and legal requirements.

The European legislation governing the free movement of new machinery within the European market is the [Machinery Directive 2006/42/EC](#) (MD). The Directive sets out the mandatory Essential Health and Safety Requirements (EHSRs) for machinery while detailed technical specifications for fulfilling these requirements are given in European harmonized standards.

Prevention through Design (PtD), Safe-by-Design (SbD) or Safety Integration (SI) are similar concepts that refer to design out hazards or minimize risks early in the design process. Principles of Safety Integration - sometimes referred to as SbD - are an essential section of the MD and guide the safe design and construction of new machinery. Risk assessment is a basic requirement of the MD and also the key tool for the safe design of machinery. However harmonized standards are not available to facilitate the process of risk assessment of new ENMs & NEPs manufacturing machinery.



Project PLATFORM is a H2020 Research and Innovation action (GA 646307) aimed to operate, in the short-term, three new Pilot Plants (PPPs) for the industrial production and commercialization of NEPs (buckypapers, treated prepregs, doped veils), for the aeronautics and automotive European industries. PPPs are not required to comply with the provisions of the MD until they are put into service in 2020 (beyond project completion), but at that time, all applicable requirements of the MD will be mandatory to PPPs.

This paper shows the methodological approach followed by PLATFORM for the safe design and putting into service of PPPs according to the EHSRs of the MD, in order to prevent and reduce the risks to health from hazardous substances emitted by PPPs (airborne emissions, wastewaters, wastes), facilitate the CE marking in 2020 and avoid potential economic costs associated with future re-adaptations or modifications needed to ensure compliance with the MD when PPPs are putting into service.

The main challenge has been the integration of all nanosafety issues in the well-established risk assessment process established by the MD. The risk assessment of PPPs has been facilitated by PLATFORM – SbD tool, a simple and friendly Microsoft Excel tool developed by the project. Since the commissioning of PPPs involves design aspects that go beyond nanosafety issues, this tool can be applied to the overall risk assessment of PPPs or only for risks related to the use and handling of ENMs and NEPs.

## **IMPLEMENTATION OF SAFE BY DESIGN STRATEGIES IN GUIDENANO TEXTILE CASE STUDY**

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GUIDEnano has the objective to develop an innovative web-based Guidance Tool to provide quantitative output for the risk assessment of nano-enabled products along their whole life cycle. Different case studies proposed by the GUIDEnano industrial partners were investigated by the different WPs to obtain information on released nanomaterials (NM), human and ecotoxicity and fate. Based on the risk assessment results, the GUIDEnano Tool will suggest risk mitigation measures (RMM) when necessary, in order to manage and decrease the risks envisaged. RMM include engineering controls, personal protective equipment and Safe by design (SbD) strategies, as an alternative way to decrease or avoid risks identified.

For the textile Case Study, owned by the company INOTEX, SbD strategies were proposed to obtain a better compatibilization of Ag NM within a cotton matrix, with the aim of reducing NM release from the textile and to maintain the functionality of the nano-enabled product. It has been described in literature that Ag NM adsorbed on cotton fibres get in contact with the skin during the wearing of the textile, and might be partially washed off into the sewage system during household washings. Therefore SbD strategies proposed were aimed to reduce Ag release from the nano-enabled fabrics and improve, as a consequence, the duration and efficacy of the textile antibacterial property. Moreover SbD were tailored according to the production processes. Specifically in this case, Ag NM must resist to the high temperature used during the textile finishing process.

A first SbD strategy focused on the application of an hydrophilic surface coating of tannic acid (TA) and mercaptosuccinic acid (MSA) on Ag NM, to improve the adhesion between NM and textile surface. In addition, a second SbD strategy focused on Ag NM shape modification to improve the NM entanglement within the cotton substrate. To this aim two Ag nano-wires with different lengths, were proposed. All textile manufacturing processes were performed at INOTEX facilities and the samples generated during the whole experiment were tested by the relevant WPs to evaluate the SbD strategies under different perspective, as reported in table 1.

Table 1 - Samples generated in INOTEX case study and relevant WPs for experimental activities.

Sample generated	Relevant WP	Experimental activity
Pristine Ag NM dispersion	Risk management	NM characterization (size, shape, NM concentration and ion content)
Treatment bath after textile impregnation	Risk management	NM characterization (size, shape, NM concentration and ion content)

	Environmental fate	NM sedimentation and aggregation
	Hazard assessment (ecotoxicity)	Ecotoxicity tests.
Textile functionalized (not subjected to washing)	Risk management	Antibacterial test on textile
	Risk management	Simulation tests for contact with sweat solution and NM characterization (size, shape, NM concentration and ion content)
Textile functionalized after 1 and washing cycles	NM release	Simulation tests for household washings and NM characterization (size, shape, NM concentration and ion content)
	Risk management	Antibacterial test on textile
	Risk management	Simulation tests for contact with sweat solution (size, shape, NM concentration and ion content).
Water from laundering collected from 1,2 and 3 washing cycles	NM release	Characterization (size, shape, NM concentration and ion content).

The rationale for the evaluation of the SbD strategies by the relevant WPs will be presented. The data collected from the samples generated in INOTEX case study will be discussed, focusing on NM release and risk management experiments.

The authors gratefully acknowledge the support of this research by the European Commission within the Seventh Framework Programme (FP7/2007-2013), Grant Agreement 604387 (GUIDEnano).